LYMPHOCYTE ANTIGENS IN SHEEP

By

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C O N T E N T S

ABSTRACT

This thesis describes a study of lymphocyte antigens in sheep. Chapter 1 reviews the literature relevant to this study and concludes with a model of what might be expected in sheep.

Chapter 2 describes the development of a suitable procedure for the cryopreservation of sheep lymphocytes. The method developed was quick, simple and reproducible. It consistently produced lymphocyte preparations with a viability greater than 95%.

Chapter 3 describes how a microlymphocytotoxicity test was adapted for use in sheep. The reproducibility of the modified test was approximately 95%. There was essentially no background cytotoxicity in control preparations.

Chapter 4 details how suitable antisera were produced. A total of 112 antisera were obtained by skin grafting, by lymphocyte immunisations and from parous ewes.

Chapter 5 describes how 17 lymphocyte antigens were defined. In 58 families with a total of 97 offspring, the antigens were shown to behave as autosomal dominants.

In Chapter 6 the relationships between antigens were investigated. They behaved as codominant alleles of one genetic system. It is proposed that the 17 antigens form part of the major histocompatibility complex in sheep.

CHAPTER 1

LITERATURE REVIEW

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Introduction

The purpose of my thesis was to determine if a major histocompatibility complex (MHC) existed in sheep and to study its organisation and structure in this species. In most species studied the most convenient target cell for detecting the presence of MHC encoded antigens is the lymphocyte. The most notable exception is, of course, the mouse. In this species antigens are also present on red blood cells. Previous work in sheep (Ford, 1973) suggested that sheep red blood cells were not suitable target cells in this species. The problem then became, firstly to study lymphocyte antigens in sheep, secondly to determine, if possible, the mechanisms of genetic control and finally to decide if there is any similarity in genetic organisation, with the genes controlling lymphocyte antigens in other species. The ultimate aim in this line of work is to determine if there are any associations between sheep lymphocyte antigens and disease resistance or susceptibility. The hope is that strong associations with diseases will allow the breeding of sheep with increased resistance to specific diseases.

Most comparative reviews of the MHC have discussed the different species separately. In this introduction, I have tried to synthesise the information from different species as I wish to emphasise the areas of similarity and also of dissimilarity between species.

Specific problems concerning e.g. freezing of lymphocytes, methods of testing for lymphocyte antigens and method of analysis have been discussed elsewhere in the thesis.

An assumption made in this review is that the properties of the different allelic products of a locus are the same unless shown otherwise. Thus, if the product of one allele has been shown to exist in serum, I have assumed that the products of all the alleles of that locus will also exist in serum.

Lymphocyte antigens have been detected in a variety of species (Vaiman, 1980). In the majority of cases where the genetic control is known, lymphocyte antigens appear to be encoded for by genes in the major histocompatibility complex of the species. Lymphocyte alloantigens not coded for by the MHC also exist. They have been found in e.g. rodents (Gasser, 1977), humans (Mayr, 1978) and sheep (Millot, 1978). Non-MHC lymphocyte alloantigens have not been considered further.

A. Classes of Loci within the MHC

Work in different species has shown that there is a remarkable similarity in the structure and function of the MHC in all higher species of vertebrates so far studied. The similarity between species is much greater than the similarity of different loci within species. This has led to the concept of classes of loci in the MHC (Klein, 1977). Each class includes genes which are structurally, and presumably functionally, similar. Klein refers to Class 1, Class 2 and Class 3 regions. The term 'regions' is used by mouse immunogeneticists to define areas of chromosome which contain the relevant loci. This is a constraint imposed upon them by their genetic material. I have used the term 'classes of loci' as it appears more appropriate in outbred species. Klein himself is well aware of the possible misuse of the terms 'region' (Klein, 1979a). In discussing work with inbred animals I will not use the term/

term 'region' unless it is genetically appropriate as it can cause unnecessary ambiguity and complexity. Each class is discussed separately.

B. Class 1 Loci

Class 1 loci correspond to the older designation 'SD loci' (Bach et al., 1976). The SD term meant serologically defined. This usage is now obsolete as other MHC loci have also been serologically defined (David et al., 1973a). Class 1 loci produce cell surface antigens which are readily detectable serologically. They are usually the first components of any MHC to be defined. My work has been exclusively confined to class 1 loci. The original definition of class 1 loci only allowed for the existence of two class 1 loci per species. Subsequently in several species a third locus has been discovered. The products of the third locus show many similarities to the products of the first two loci. Consequently, I have treated the third locus as a class 1 locus. I have assumed that results found for the first two loci will also apply to the third locus, although in several instances this has not been shown.

1. Existence of Lymphocyte Alloantigens

Differences in leucocyte antigens between members of the same species can be readily shown to exist. The production of anti-leucocyte antibodies following alloimmunoisation provides evidence for allogenic differences. An alternative approach is to show that leucocyte samples from different individuals differ in their response to an antiserum/

antiserum or preferably, several antisera. If such a test is properly controlled it too can show allogenic differences between leucocytes. These and similar methods have been used to demonstrate allogenic differences in white cell antigens in a variety of species. These include mouse (Amos, 1953), man (Dausset, 1958), rabbit (Terasaki et al., 1961), chicken (Terasaki et al., 1959), rat (Bogden and Aptekman, 1960), dog (Altman and Simonsen, 1964; Puza et al., 1964), rhesus monkey (Balner et al., 1965b), chimpanzee (Shulman et al., 1965), cattle (Spooner et al., 1978), pig (Vaiman et al., 1970b) and sheep (Millot, 1971).

The detection of specific lymphocyte antigens has been achieved in a variety of tests in a number of species. Serological studies on the class 1 antigens of the MHC have shown that there is great similarity between species. Unfortunately this similarity does not extend to nomenclature. Nomenclature is of two types, haplotype nomenclature and individual antigen nomenclature. Haplotype nomenclature has only been used in species where inbred strains exist. This is described later. Individual antigen nomenclature is described in the next section when the different antigen systems are discussed.

2. Antigen Systems

i) Inbred Species

In mice, the MHC encoded antigens are present on both red blood cells and lymphocytes (reviewed by Edidin, 1972). Haemagglutination tests have been the main procedure used to detect the class 1 antigens of the MHC in this species. Over seventy antigens in the MHC complex have been found. Klein (1975) reviews the field up to 1974.

Several/

Several antigens have been found since then. Each antigen discovered is given a number in sequence e.g. H-2.1, H-2.2, H-2.3, etc. Antigens discovered in wild mice are given a number in sequence starting from 101 (Klein, 1972). Thus, H-2.101, H-2.102, etc. There appears to be two types of class 1 antigens present. They are called public and private antigens. Public antigens are shared between haplotypes (Klein, 1971). Private antigens are haplotype specific (Hoecker et al., 1954). The use of the terms 'private antigen' and 'public antigen' bears no relation to their use in red cell serology.

Certain antigens can be arranged into mutually exclusive series, apparently controlled by alleles at two genetic loci (Snell et al., 1971). The two loci are called H-2D and H-2K. There is also a third gene coding for class 1 molecules in the H-2 complex. This third gene is called H-2L (reviewed by Démant and Neauport - Sautes, 1978). The alleles of genes of the H-2 complex are sometimes referred to by the gene designation followed by the superscript of the relevant haplotype e.g. $H\text{-}2D^{k}$ can refer to the H-2D allele of the K haplotype. These three genes are in very close linkage on chromosome 17. The gene products are probably expressed codominantly. (Klein, 1975). More than three antigens can occur on one haplotype of the H-2 system. There are several explanations for the observed serological complexity. Firstly the complexity may be an artefact caused by the method of analysis. Secondly, the antigens present may reflect the existence of a large number of genes. Thirdly, there may be a small number of genes but different gene products may each carry more than one antigen. The third hypothesis is supported by the observation that public and private antigens are probably present on the same molecule (Hauptfeld and Klein, 1975).

In both rats (Bodgen and Aptekman, 1960) and chickens (Schiermann and Nordskog, 1962) MHC encoded antigens have been shown to be present on both red blood cells and on lymphocytes. In these species haemagglutination tests have been the main procedure to detect MHC antigens. As in the mouse, the gentic control of lymphocyte antigens is inferred from the study of the antigens on red blood cells. In these two species, the presence of inbred lines has meant that the serological analysis of MHC class 1 antigens has followed the mouse model. In both rats and chickens, several antigenic specificities are coded for by each haplotype. Unlike the mouse, these specificities have so far been arranged into only one series. Nonetheless recombinations between class 1 antigens have been serologically detected in chickens (Schiermann and McBride, 1969; Hála et al., 1975, 1976).

The MHC of the chicken is called by B system. The alleles are designated by superscripts e.g. B^1, B^2, while the "complex" or "total" antigens are denoted by subscripts e.g. B_1, B_2, etc. For a review of the system see Pazderka et al. (1975) and Hála (1977).

In the rat, the presence of two class 1 loci has been proposed by Dewitt and McCullough (1977). They produced an antiserum to a specificity which appeared to be linked to AgB-3. Further, they detected a possible recombination event in one of thirty-nine offspring. Further breeding studies appeared to confirm this hypothesis. They called the two loci AgB-A and AgB-B. Gill and Kunz (1976) also concluded that there might be two class 1 loci in the rat. This conclusion was based on an analysis of strain reaction patterns. Similarly,/

Similarly, Sporer et al. (1979a,b) claim to have identified two class 1 loci by biochemical analysis of the gene product. Also, Natori et al. (1979) claimed to have identified two different gene products by sequential immunoprecipitation. Nonetheless, the presence of two class 1 loci in the rat has not been generally accepted. The term AgB-B has subsequently been used to refer to a region which codes for Ia antigens, Ir genes and MLR response but not class 1 loci. (Günther et al., 1978). For a recent review of the rat system see Günther and Stark (1977).

The putative guinea pig MHC is called GPLA in analogy with the human lymphocyte system A (HLA). Four lymphocyte alloantigens have been detected in guinea pigs (Sato and de Weck, 1972; Geczy and de Weck, 1977). The four antigens are currently designated B.1, B.2, B.3 and B.4. So far no animal has been found to carry more than two of the four specificities. The four antigens are therefore considered to be the products of four alleles at a single locus. An additional specificity (S.1) has also been detected by certain anti-B.1 sera. Geczy and de Weck (1977) believe that S.1 might be the product of a second locus. As yet S.1 has not been found in the absence of B.1 and the sera cannot be purified by absorption.

There are very few fully inbred strains of guinea pigs. Only two NIH 2 and NIH 13, have been used in guinea pig typing. The two strains are both B.1 positive and B.2, B.3 and B.4 negative. Haplotype nomenclature is not used. It would be unnecessary with only two inbred strains.

Typing of outbred guinea pigs revealed that a small number of animals were B.1, B.2, B.3 and B.4 negative. It therefore appears that not all specificities can be detected at present. The GPLA system has been reviewed by Geczy and de Weck (1977).

No lymphocyte alloantigens have so far been detected in Syrian hamsters (Duncan and Streilein, 1978a,b). A variety of different alloimmunisation and xenoimmunisation procedures (rabbit anti-hamster and rat anti-hamster) have failed to produce sera capable of detecting alloantigenic differences. The presence of an MHC is inferred from the observation that genes responsible for skin graft reactivity, graft versus host reaction and mixed lymphocyte response are closely linked (Duncan and Streilein, 1978b). The putative MHC is called Hm-1.

Hamsters in captivity are all derived from just three animals captured in Syria (Adler, 1948). Further, the three animals were possibly litter-mates. If the three animals were identical for class 1 loci, this would explain the failure to detect lymphocyte alloantigens serologically. Recently Duncan and Streilein (1978b) have raised alloantibodies by immunising Syrian hamsters derived from wild populations. Preliminary results suggest that the alloantibodies may be capable of detecting lymphocyte alloantigens. The Hm-1 system has been reviewed by Duncan and Streilein (1978a,b).

Three different notations have been used to describe the proposed rabbit MHC. They are RLC (Black, 1967), RbH-1 (Chai, 1974) and RL-A (Matej, 1970b; Ehlers and Ahrons, 1971; Tissot and Cohen, 1972).

RL-A/

RL-A is the term most commonly used. Groups of sera have been used to define nine different alleles at the RL-A locus (Tissot and Cohen, 1974). The patterns of reactivity with all sera rather than individual serum reactions were used to define alleles. The alleles have been designated RL-A^a, RL-A^b, RL-A^c, etc. In general, the serological approach adopted follows the mouse H-2 model. Fully inbred lines do not yet exist in rabbits and partially inbred lines have been used instead. A brief review of the rabbit MHC has recently been carried out by Iványi (1977b).

ii) Outbred Species

The human MHC is called HLA. The HLA system has been reviewed by several authors under the editorship of W. F. Bodmer (1978) and by Albert and Gotze (1977). Additional reviews include those by Mayr (1978), Bach and van Rood (1976a, b,c) and Amos and Ward (1975). The development of the system has been very different from that of the mouse MHC. The HLA system is not readily detected on red blood cells. The cell type which is most commonly used to detect HLA antigens is the lymphocyte. Although leucocytes are occasionally used. The detection of an antigen system in an outbred population initially followed the model developed by workers of the red blood cell groups. A description of the approach taken in studying red blood cells is given by Race and Sanger (1975). However, initial difficulties prevented the full application of this approach. The low titre of many antisera and the difficulty in obtaining white blood cells in large quantities made absorption difficult to perform. Consequently, classical cross-absorption techniques were seldom used to purify antisera.

Initial tests to detect antigens mainly involved agglutination procedures. These have largely been superseded by cytotoxicity tests. Lymphocytotoxicity tests were developed in mice by Gorer and O'Gorman (1956). They were adapted for small scale use (microlymphocytotoxicity tests - mlct) in humans by Terasaki and McClelland (1964). The test was subsequently modified by Mittal et al. (1968). The majority of investigators use the NIH technique (NIAID Manual of Tissue Typing Techniques, 1976). Although other modifications of the procedure are used (e.g. Kissmeyer-Nielsen and Kjerbye, 1967). Leucoagglutination procedures are still used, especially to detect the BW4 and BW6 antigens (van Rood and van Leeuwen, 1963). Complement fixation tests are also used (Colombani et al., 1967; D'Amaro, 1970; Colombani et al., 1971). Usually, complement fixation tests are used in conjunction with mlct tests to confirm antigen assignment.

Sera can come from two sources. The sources are parous women or alternatively recipients of allogeneic tissue (blood transfusion, volunteers immunised with skin grafts, kidney allograft recipients). Xenogeneic antisera have also been used but less extensively. The source of sera and methods used to detect antigen-antibody reactions are more fully discussed later in the thesis.

Due to the difficulties in obtaining suitable reagents and the irreproducibility of the tests employed, a statistical approach was initially used to define antigens. This procedure was pioneered for white cell antigens by van Rood and van Leeuwen (1963) and developed by Payne et al. (1964). The statistical method has been discussed in Chapter 5.

The statistical approach is not now so extensively used in HLA typing. The presence of well-characterised sera to detect many of the known antigens makes statistical analysis less necessary. Nonetheless the method remains an excellent procedure for the initial characterisation of unknown sera.

The development of the HLA system has been greatly assisted by a series of Workshops (Balner et al., 1965a; Curtoni et al., 1967; Terasaki, 1970; Dausset and Colombani, 1973; Kissmeyer-Nielsen, 1975; Bodmer et al., 1978). These workshops have been attended by most of the scientists researching HLA throughout the world.

Historically, individual antigens were defined by several groups. Dausset (1958) defined the 'Mac' antigen (now HLA-A2) in the French population. Subsequently van Rood and van Leeuwen (1963) defined 4a and 4b (now Bw4 and Bw6). Payne et al. (1964) described three antigens in Americans, LA1, LA2 and LA3. The antigens all behaved as autosomal codominants.

The first allelic relationship between lymphocyte antigens in man was found by van Rood and van Leeuwen (1963). This involved the 4a and 4b specificities. The authors cited several observations in favour of the assumption of allelism. Firstly, the observed phenotype frequencies showed good agreement with those predicted by Hardy-Weinberg in a group of 347 unrelated humans. Further confirmation came from the observation that the donors of the sera used (all parous women) were homozygous for the opposite specificity while their husbands possessed the necessary stimulating antigen. Family studies involving a total of 140 offspring did not contradict the hypothesis and the observed and expected distribution of offspring were in close agreement. Also the authors found/

found a definite dosage effect. Cells from homozygous individuals were more strongly agglutinated and by higher dilutions of the antisera than were the cells from heterozygous individuals. Additionally, the authors found significant X^2 values between sera which detected the putative alleles. Bodmer et al. (1969) subsequently showed that significant X^2 values which are caused by an excess of discrepant reactions (the frequency of +- and -+ exceeding the frequency of ++ and -- reactions) are almost certainly caused by allelism or by very closely linked genes in repulsion. Although not presented as evidence by the authors, the X^2 values between the two different groups of sera support the assumption of allelism.

The human class 1 antigens are under the control of three different but closely linked loci. Antigens at the first two loci were the first to be detected. Serological evidence for the presence of two closely linked loci came from a variety of sources. The known alleles could be arranged in two series (Payne et al., 1964; van Rood and van Leeuwen, 1963). As these two series were inherited together in family studies, this led to the concept of two closely linked loci with multiple alleles (Dausset et al., 1967). The detection of recombinant offspring (Kissmeyer-Nielsen et al., 1969) lent support to the two locus hypothesis. The concept of two loci was generally accepted in 1970 (Joint Report, 1970). The two loci were called HLA-A and HLA-B.

Serological evidence for a third locus (HLA-C) was presented by Sandberg et al. (1970), by Solheim and Thorsby (1973) and by Mayr et al. (1973). Confirmation came in 1974, Low et al. (1974) reported finding a recombinant between HLA-B and HLA-C. Subsequently, recombination between HLA-A and HLA-C has also been detected (Waltz and Rose, 1977).

Elegant confirmation of the existence of three genes came from Bernoco et al. (1973). They demonstrated the independent movement on the cell surface of the gene products of these three loci. Subsequently, confirmation of the three gene hypothesis has come from biochemical studies (reviewed by Barnstable et al., 1978).

In men, as in mice, several specificities appear to be present on the same molecule. In particular, Ayres and Cresswell (1976) have found that the antigenic determinants B7 and Bw6 are carried by the same molecule. Similarly, B12 and Bw4 are also present on the same molecule. B12 has now been split into Bw44 and Bw45. Usually, Bw44 is inherited with Bw4, while Bw45 is inherited with Bw6.

Nomenclature is defined by the WHO nomenclature committee. This committee generally meets after each International Histocompatibility Workshop. The latest report was in 1977(WHO report, 1977). The different loci in the HLA system are given a letter in alphabetical order following the sequence of discovery. The three class 1 loci are called HLA-A, HLA-B, HLA-C. HLA-D is used for a class 2 locus. Each antigen is identified by the letter of the locus which controls it, followed by a number defining the specificity at that locus (e.g. HLA-A1, HLA-A2, HLA-B7, HLA-B8). The symbol 'w' preceding an antigen means that it is still provisionally defined (e.g. HLA-Bw51, HLA-Bw52). Roman type is used for antigens and italic type for gene symbols (including haplotypes, alleles and loci). Superscripts and subscripts are not used. For historical reasons there is no overlap in the numbers assigned to the HLA-A and HLA-B loci. In HLA-C, the numbers have been assigned sequentially. The w symbol is retained before the HLA-C series, although several of these antigens are by now well defined. This/

This is to avoid confusion with the complement components (C1, C2, C3, etc.). This nomenclature is under review. Joysey and Wolf (1978) have listed the specificities known by 1978 at the HLA-A, HLA-B and HLA-C loci.

The study of the MHC in outbred animals has followed the methods used in the study of the HLA system. Recent reviews have been provided by Vaiman (1980) and by several authors under the editorship of Götze (1977).

In chimpanzees initial trials with leucoagglutination techniques have been replaced by microlymphocytotoxicity tests. One stage tests are used. A further discussion on one stage and two stage tests is given in Chapter 3. Antisera are used at the optimal dilutions to reduce the number of specificities detected by the sera. Similarly antisera are absorbed with leucocytes and/or platelets to improve the quality of sera. Certain sera are also cross-absorbed to determine the number of specificities present. Antisera are raised by deliberate immunisation. Initially immunisation was carried out randomly. Once antigens were defined, donors and recipients were deliberately matched to produce antisera against required specificities. This in turn led to better matching which has led to production of higher quality sera (Balner, 1977). Two loci coding for class 1 antigens have been detected (Balner et al., 1974a,b). Evidence for two series of antigens comes from both family and population data. Eleven antigens were defined and X^2 tests for independence and allelism were made on the population data. X^2 tests for independence were inconclusive. The authors attributed this to the small population size. X^2 values for allelism were more informative. There were several significant positive associations between pairs of antigens. This indicated that the specificities were unlikely/

unlikely to be part of a single allelic series. The absence of 'triplets' (an animal positive for three specificities) within any one series was consistent with the two locus hypothesis. Additionally, within each series, the observed and expected phenotype combination of specificities was compared. The observed fit for both series was consistent with the expectations of Hardy-Weinberg equilibrium.

Family data were also consistent with the two-locus hypothesis. However no recombinants were observed. Therefore family studies were unable to discriminate between a one or two locus model. Immunogenetic analysis of further population data supported the two locus hypothesis (Balner et al., 1978). Recombinants have not yet been observed. In the same paper, Balner et al. (1978) tentatively suggested the existence of a third locus.

Serological analysis has suggested the existence of similar antigens in chimpanzees and man (Balner, 1977). This is discussed later. The Bw4 and Bw6 supertypic specificities are amongst those common to chimps and man. Their existence in chimps would suggest the presence of two or more antigens on certain class 1 molecules.

In rhesus monkeys, the procedures used have closely followed the human model. The MHC in this species is called RhLA. Rogentine et al. (1971) postulated that at least two loci were necessary to account for the phenotypic distribution and segregation patterns of the three antigens that they were able to detect. Only one instance of recombination has been demonstrated between the two loci (van Vreeswijk et al., 1977). Therefore the low loci are apparently very closely linked. The two loci are called RhLA-A and RhLA-B. A total of thirteen/

thirteen alleles have been detected at the RhLA-A locus and twelve at RhLA-B. The gene frequency of undetected antigens (null alleles or blanks) is estimated at 0.099 at RhLA-A and 0.155 at RhLA-B (Balner, 1977).

A limited amount of work has been carried out on other primate species. This has been reviewed by Balner (1977). In the same article, the MHC of chimpanzees and rhesus monkeys is also reviewed.

In dogs, Vriesendorp et al. (1971) have shown that most of the leucocyte specificities detected were controlled by one genetic system. This system was thought to be the dog MHC and is called DLA. Three loci, DLA-A, DLA-B and DLA-C are thought to be involved in the genetic control of leucocyte alloantigens. A low frequency of recombination between DLA-A and DLA-B has been reported (Vriesendorp et al., 1973). Recombination involving DLA-C has not yet been found. Currently, a total of seven alleles at DLA-A, six alleles at DLA-B and three alleles at DLA-C has been found. The frequency of 'blanks' is still considerable (Vriesendorp et al., 1977). For each locus, the frequency of 'blanks' lies between 30% and 60%. The DLA system has been reviewed by Vriesendorp et al. (1977).

A number of workers are involved in the study of lymphocyte antigens in cattle. Nine laboratories contributed antisera to an international comparison test in 1978. The sera were tested in one laboratory against 130 largely unrelated cattle (Spooner et al., 1979). Eleven antigens were defined by the sera. The population data was consistent with the specificities representing eleven alleles controlled by a single genetic system. Family studies have been presented elsewhere. (Spooner et al., 1978; Amorena and Stone, 1978; Caldwell et al., 1977). They too are consistent/

consistent with control of bovine lymphocyte antigens by a single genetic system. The putative genetic system is thought to be the bovine MHC. The system is called BoLA. The agreed specificities are given numbers. As in the human, the letter w denotes workshop specificities. Specificities detected by individual laboratories and not yet confirmed are given laboratory designations e.g. EDC1, EDC2, TXC1, TXC2. The C stands for cluster defined specificities, TX for Texas and ED for Edinburgh.

In pigs, the MHC is called SLA (Vaiman et al., 1970b) or PLA (Viza et al., 1970). Vaiman (1979) has claimed to have identified three loci controlling class 1 antigens on lymphocytes. The evidence for three loci comes from serological and biochemical studies. Also, lysostrip experiments have confirmed the independent of three specificities on a single haplotype. However this evidence has yet to be published. To date twenty-two specificities have been recognised. The SLA system has been reviewed briefly by Vaiman (1979) and by Iványi (1977a).

Three laboratories have published work on sheep lymphocyte antigens. Ford (1974, 1975) used twenty-two antisera to identify twenty-one possible leucocyte antigens in a variety of breeds. He was able to state that the detected antigens were found in both males and females and were not therefore Y-linked. However, he was unable to decide between four models of genetic control, namely -

i) single locus with many alleles,

ii) multiple closely linked loci,

iii) loci which are not closely linked,

iv) loci on different chromosomes.

Schmid et al. (1975) used thirty-one antisera to define thirty-one antigens in German Merino Landrace sheep. The specificity of the antisera was narrowed and checked by cross-absorption. The thirty-one antigens behaved as if controlled by codominant genes in family studies. Unfortunately, no attempt was made to ascertain relationships between the different antigens.

Finally, Millot (1978) used nine antisera to define eleven specificities in the French prealpe breed. He argued that four loci coded for the eleven antigens. OLA-A coded for five alleles and OLA-B for four alleles. The two loci were closely linked. Only two recombinants were detected in 295 informative meioses. One of the other two specificities was controlled by an unlinked locus which he called OL-Z, the other by a loosely linked locus which he called OL-X.

The sera used in the three sheep studies were kindly made available to me by the authors. The results obtained have been presented in Chapters 4 and 5.

In horses, Bailey et al. (1979) claimed to have detected fourteen lymphocyte alloantigens. They have presented data on only four specificities, E1, E2, E3 and E4. In one sire family E1 and E2 segregated from the sire as alleles while in another family E3 and E4 segregated as alleles. Also, in 204 horses typed, no animal possessed more than two of the four specificities. Bailey et al. have suggested that the four markers are coded for by a series of codominant alleles at one locus. They have called the locus ELA.

In the South African clawed toad (Xenopus laevis), Du Pasquier et al. (1975) looked at fifty-six siblings. They raised antisera to red blood cell antigens by inter-sibling skin grafting and blood transfusions. Antisera were purified by absorption with appropriate siblings. After absorption the antisera were apparently specific for red blood cell antigens which segregated with lymphocyte activating determinants. Further, they looked at skin graft rejection. The time of rejection depended upon the haplotype disparity as determined by red cell antigens and the results of mixed lymphocyte culture. These observations strongly suggest that the red cell antigens are homologous to MHC tissue antigens detected in other species. They also suggest that Xenopus laevis (a higher amphibian) has an MHC-equivalent.

In summary, the study of lymphocyte antigens in different species has revealed:-

i) the majority of lymphocyte antigens are controlled by a single genetic system.

ii) there are many different antigens in the system. In the species where only a few antigens are defined, there is either a restricted genetic origin of the animals or the majority of antigens are not defined. The question of polymorphism is considered later.

iii) In inbred strains, several specificities are assigned to haplotypes. There are often more specificities on a haplotype than different loci. This could be due to different antisera detecting the same specificity or to several antigens occurring together on the same molecule. Single molecules have been shown to carry more than one antigen in both mice and men.

In most outbred species, alleles are recognised by single antigens. This may be a consequence of the procedure used for screening antisera before use. Sera which react with a determinant common to two or more gene products will be 'split' by sera which only react with a determinant found on only one gene product. Generally, the 'broad' sera (the sera which react with more than one gene product) are not used for antigen definition. There is one problem with this approach which does not appear to be fully appreciated.

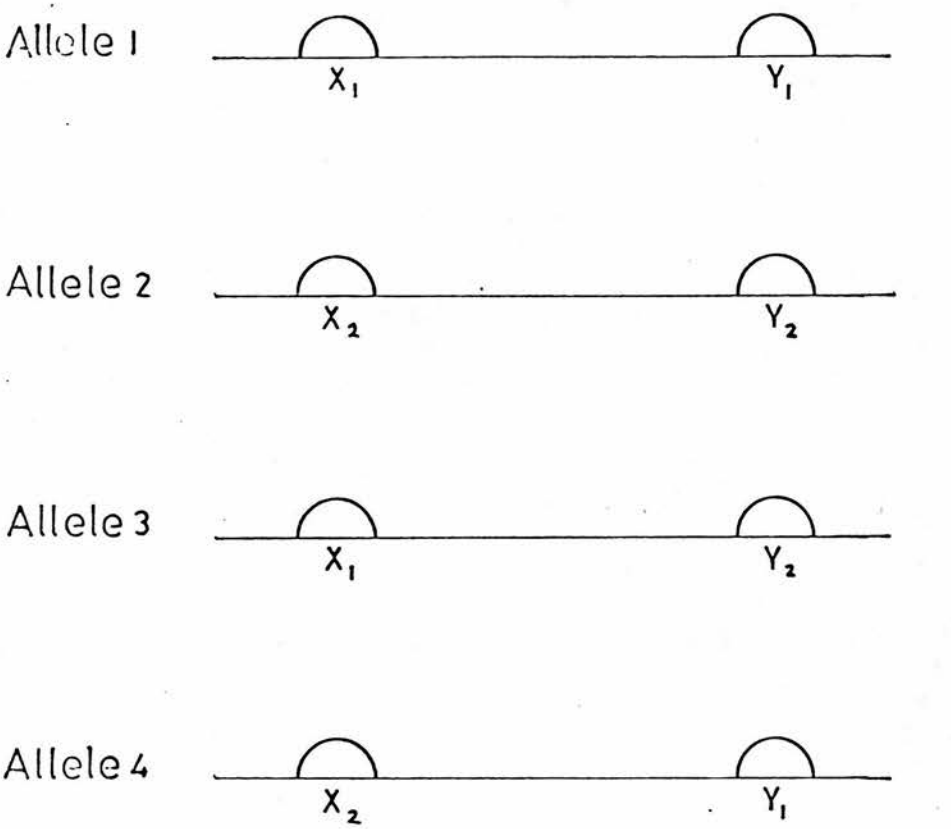
If there are two antigenic determinants on one molecule (say x and y), and if the two antigenic determinants are polymorphic (say x_1, x_2 and y_1,y_2) there are four possible allelic products (Figure 1). if only one determinant is used to detect an allelic product, then the two 'gene products' detected will actually be heterogenous groupings. This will not show up in family studies as both 'gene product' classes will show regular inheritance. If a locus has multiple alleles this problem could be magnified by several allelic products sharing a common determinant. The size of the problem will be determined by the relative frequency of the allelic products which share common determinants.

As mutation and intragenic recombination are very rare (Whitehouse, 1965) it is possible that two types (x_1, y_2 and x_2y_1) may not occur at all.

The number of loci found within the system varied. Three loci have been found in man, mice, pigs and dogs. The most convincing evidence for three loci comes from the independent distribution of three antigens from the same haplotype on the cell membrane as observed by Bernoco et al. (1973) in the human. Serological evidence is less convincing. Recombinants between two of the three loci have been detected in men, mice,/

Figure 1

Diagram Representing the Four Possible Alleles When a Molecule Carries Two Polymorphic Antigenic Determinants



mice, pigs and dogs. However, recombinants involving the third locus have so far only been found in man. As crossing over can take place within genes (Whitehouse, 1965) as well as between them, the discovery of a recombinant haplotype does not establish that different loci are involved. Intragenic recombination is however very much less frequent than intergenic recombination. Therefore, if the frequency of recombination is known, this value could be used to establish the existence of separate or single loci. Quoted values for recombination frequencies are about 0.8% in humans (Bodmer, 1972), 0.5% in mice (Shreffler and David, 1975), between 0.1 and 2.2% in dogs (Vriesendorp et al., 1973), 0.05% in pigs (Vaiman, 1978), 0.3% in rhesus monkeys (van Vreeswijk et al., 1977) and 0.67% in sheep (Millot, 1978). Recombination frequencies of this order suggest intergenic rather than intragenic recombination. Thus, the existence of at least two loci appears likely. However, the results should be treated with caution. It is not clear if only informative matings have been used to calculate recombination frequencies. Certain matings will be uninformative e.g. if the parents share antigens, or if either parent is a homozygote or if not all antigens can be detected at a locus. The use of uninformative matings will lead to artificially low values of recombination. Also, if recombination is looked for and not found, this is not usually reported. This will lead to artificially high values for recombination. Finally, only a very small number of recombinants have been found, e.g. Vaiman (1978) has only found one recombinant pig. Millot (1978) has only found two recombinant sheep. Therefore it would appear that the existence of recombination alone is insufficient to determine that different loci are involved.

Also, given that one molecule can carry more than one antigen, it is probably also unsafe to assume the number of alleles on a haplotype is equal to the number of different loci involved. Therefore, the existence of separate loci cannot be regarded as proved when only serological evidence is presented.

A good fit to Hardy-Weinberg has also been used to state that only one locus is involved (e.g. J. G. Bodmer, 1978). This test also depends upon the total gene frequencies of the known alleles. If the frequency of unknown genes is high, then testing for Hardy-Weinberg is not a very stringent test of how many loci are involved.

However, biochemical and serological evidence obtained in men and mice makes it very likely that there are three class 1 loci in the MHC. Thus, it is reasonable to assume that three loci are involved in all mammalian species. The validity of assuming similar models of genetic control for different species will depend upon the homology between species. Evidence for interspecies homology comes from a variety of sources. These have been discussed below.

The absence of triplets (animals possessing three or more specificities) has also been taken as evidence that only one locus is involved, e.g. Bailey et al. (1979) with horses, Gaczy and de Weck (1977) with guinea pigs. In horses and guinea pigs only four alleles have so far been reported. The frequency of the unknown ('blank') alleles was not given, but if the frequency of unknown antigens was high, this could account for the absence of triplets. Alternatively, the antigens could all be products of one locus. This could be a chance effect due to the small numbers involved or the products of different loci may be of different immunogenicity. In humans, there is evidence to suppose/

suppose that the products of the third locus, HLA-C, are less immunogenic than the products of HLA-A and B, (Ferrara et al., 1975, 1978a). However in the well studied species, (e.g. mouse, man, chimpanzee, rhesus monkeys), once the second locus was established, the known alleles were apportioned approximately equally between both loci. This would suggest that the first two loci (A and B) do not differ in immunogenicity.

3. Cross-reactivity

One of the major technical problems in HLA serology is the problem of cross-reactivity. Antisera to leucocyte antigens frequently react with more than one HLA antigen. Often this is due to the occurrence of different antibodies in the same serum. The different antibodies can be resolved by titration and absorption experiments. Occasionally however, absorptions will remove all activity from the serum. This can happen even when the absorbing cell does not contain all the antigens detected by the serum (Mittal and Terasaki, 1972).

Also, immunisation experiments have shown that antibodies can be produced which will react against antigens not present on the immunising cell. (Thorsby et al., 1970). The observation that a single antibody population reacts with more than one antigen has been called cross-reactivity.

Cross-reactivity is a common phenomenon in HLA typing. There are several families of cross-reacting antigens. At the HLA-A locus, A1, A3, A11, form such a family as do A2 and A28. At the HLA-B locus/

locus the prominent cross-reactive groups (CREGS) are B7, B27, BW22 or B5, BW15, B18 (Mittal et al., 1972; Mueller-Eckhardt et al., 1972). The groups of cross-reacting families always involve alleles at the same locus. Cross-reactivity has been claimed in sera produced as a result of pregnancy (Ahrons, 1971) blood transfusions (Curtoni et al., 1972) or after planned immunisation (Dausset, 1970). Even in weak antisera, concentration can reveal the presence of previously undetected cross-reactions (Mueller-Eckhardt et al., 1972).

There are four possible explanations for the observed cross-reactivity.
a) There may be several antibody populations present in a single serum. This is the explanation favoured by Albert and Götze (1977), who claimed that "Titration experiments of antisera containing antibodies directed against a group of cross-reacting antigens revealed that most of these sera have one "main" specificity which is relatively high-titred (this specificity corresponds to the antigen present on the immunising cells) while the other "cross-reacting" specificities generally have a low titre (Albert et al., 1973)". Absorption-elution experiments have also shown the existence of several antibody populations in sera which were apparently monospecific by classical cross-absorption criteria (Mueller-Eckhardt et al., 1972; Colombani et al., 1969, 1970). Albert and Götze (1977) believe that the different antibodies in a serum act synergistically. Absorption removes one antibody component, rendering the serum negative, although a sublytic concentration of antibodies remains in the absorbed serum.

However, if cross-reactivity is due solely to the presence of several antibodies reacting in a serum, then cross-reactivity between different antigens should be more or less random, e.g. an antibody to HLA-A11 and

HLA-A1/

HLA-A1 should be as common as an antibody to HLA-A11 and HLA-A2. Cross-reactive families are not expected. Also, if anything, cross-reactivity should be more common between loci rather than within loci. Further, this hypothesis does not explain why some antibodies act synergistically while others do not.

b) An alternative possibility is that cross-reactivity is due to antisera reacting with different antigenic determinants on the same molecule. It is possible that some of the determinants may be common to different allelic products. Cross-reacting antisera could contain antibodies against the common determinants. Under this hypothesis, some antisera will detect only the unique determinants on a given allelic product. Other antisera will detect other determinants common to more than one allelic product. It is only the latter antisera that will be cross-reactive. A number of antigenic sites on the same molecule are necessary with this hypothesis. As cross-reactivity varies, even between members of the same cross-reactive group, the same antigenic determinant cannot be common to all members of a group. Different irs of allelic products must share different antigenic determinants. Also, in certain instances cross-reactivity is unidirectional (Mittal and erasaki, 1972). Thus, antisera against antigen A will be absorbed by ells carrying antigen B but will not be absorbed by cells carrying antigen A. This unidirectionality cannot be explained by the presence of identical antigenic determinants on different molecules. Although it could possibly be due to the use of selected antisera (Mittal and Terasaki, 1972).

c) The third explanation is that cross-reactivity is due to biochemical similarity of the respective antigens. The observation that only some sera directed against an HLA antigen show cross-reactivity whereas other sera directed against the same specificity do not, has been taken as conclusive evidence against this hypothesis. This can be countered by claiming firstly, that several antigens may be present on different alleles. Secondly, given that molecules can carry more than one antigenic specificity, the different sera tecting the same HLA gene product may actually be reacting with different determinants. Only some determinants need be cross-reactive.

d) The fourth explanation is that cross-reactivity may be due to some sera containing antibodies to non-HLA antigens. The non-HLA antibodies and the anti-HLA antibodies act cumulatively. Removal of one component by absorption renders the serum negative although a sublytic amount of antibodies remain in the serum. This hypothesis has the advantage of explaining the inconsistency of cross-reactivity. Only some sera directed against an antigen show cross-reactivity. Similarly, only some sera would be expected to possess antibodies against non-HLA components. Only some cells of a given specificity absorb out a cross-reactive serum, while other cells of the same specificity do not. Only some cells would be expected to possess the non-HLA antigen. However this theory cannot easily explain the existence of cross-reactive families nor explain why cross-reactivity only occurs with the products of one locus.

Cross-reactivity can lead to false classification. For example, HLA-B7 homozygotes may react with antisera against HLA-B27. This could lead to their being wrongly labelled as HLA-B7, B27 (Joysey and Wolf, 1978).

Cross-reactivity has also been reported in other species. David et al. (1973b) and Murphy and Shreffler (1975a,b) have reported cross-reactivity in the mouse. In this species cross-reactivity apparently occurs between gene products of the H-2K locus and gene products of the H-2D locus. Balner et al. (1973b) and van Vreeswijk et al., (1977) have reported cross-reactivity in the rhesus monkey. Cross-reactivity has also been reported for dogs (Smid-Mercx et al., 1975; Joint report, 1973), chickens (Briles et al., 1957; McDermid, 1964) and rats (Günther and Stark, 1977). However, different authors mean different things when they use the term cross-reactivity.

In the rat, chicken and mouse, cross-reactivity is used to refer to e presence of shared antigens between different gene products. In the dog the term cross-reactivity is used when a serum which detects one antigen gives occasional reactions with cells carrying another antigen. The extra reactions seem to fall in definite groups. Thus, sera recognising DLA-A2, A7 or A10 frequently have extra reactions with cells carrying one of these three antigens (Vriesendorp et al., 1977). Formal absorption studies have not been done. Although, immunisations against one antigen of a cross-reactive group can produce antisera reactive with all alleles of the group, (Vriesendorp et al., 1977).

In the rhesus monkey, absorptions have been done (Balner et al., 1973b). ey indicate unidirectional cross-reactivity between the gene products of leles 2 and 14.
Both alleles belong to the RhLA-B locus.

Others have used the term cross-reactivity to refer to similarities between MHC determinants of different species (e.g. Balner, 1977). This point is discussed in more detail later in this chapter.

4. Tissue Distribution

The distribution of the class 1 MHC antigens has been studied in several species. A comparison of results indicates many similarities but also a few discrepancies. The distribution of class 1 antigens has recently been reviewed both in man (Albert and Götze, 1977) and mouse (Edidin, 1972; Klein, 1975). I have referred to these sources where appropriate. I will first discuss the antigen distribution in body fluids and on different cell types then the distribution on different tissues.

a) Antigen expression in body fluids and on different cell types

In chickens, rats, mice and Xenopus laevis red blood cells are usually used to detect class 1 antigens. In all other species lymphocytes have been used. However, the antigens detected on red blood cells are probably also present on lymphocytes. This has been shown for chickens, rats and mice (Götze, 1977).

The expression on red blood cells in different species is less certain. Antigens have been reported to be absent from mature erythrocytes in sheep (Ford, 1973) and dogs (Bull and Vriesendorp, 1977). Boroskova and Démant (1967) claimed that the S red blood cell system could be the bovine MHC. However, this is disputed (Ostrand-Rosenberg and Stormont, 1974; Folger and Hines, 1976). In man, HLA antigens occur on reticulocytes but were reported to be absent on mature cells (Harris and Zervas, 1969). However it is now thought that HLA antigens are present on mature red blood cells but in very low quantities (reviewed by Race and Sanger, 1975). They are responsible for three antigens in the Bg system. This result is not inconsistent with the mouse data.

Murine/

Murine erythrocytes carry less than 10% of the antigen present on spleen cells. It may be that all mammals have a small quantity of class 1 antigens on red blood cells. This small quantity would prevent ready detection of these antigens.

In chickens, in marked contrast to the mouse, the MHC antigens are present in much greater quantities on red blood cells than on spleen cells. This may be due to the fact that chickens red blood cells are nucleated. Nonetheless, there would appear to be a genuine difference between species.

Class 1 antigens have also been shown to be present on platelets in man (stated by Albert and Götze, 1977), rhesus monkeys (Balner, 1977), dogs (Vriesendorp et al., 1977) and rats (Davies and Alkins, 1974). Other cell types shown to carry class 1 antigens are fibroblasts in chicken embryos (Hložánek and Hála, 1969) and humans (Melief et al., 1967), human endothelial cells (Moraes and Stastny, 1975) and sheep chondrocytes (Elves, 1974). The presence of human class 1 MHC antigens on sperm is disputed (Siegler and Metzgar, 1970; Fellous and Dausset, 1970). Murine sperm apparently do not express MHC antigens (Klein, 1975). In contrast, Vaiman et al. (1978a) have found class 1 antigens on pig sperm. Mouse ova do not appear to express H-2 antigens.

Soluble HLA antigens have been found in serum (Charlton and Zmijewski, 1970), seminal plasma (Singal et al., 1971) and milk and colostrum (Dawson et al., 1974; Kachru and Mittal, 1975). They have not been found in chicken abdominal fluid (McDermid, 1964). Histocompatibility antigens have also been found in pig plasma (Chardon et al., 1977).

Swanson (1973) found that HLA B7 negative (i.e. Bg^a negative) red cells could be rendered positive by soaking them in HLA B7 positive serum. It is possible that human red blood cells absorb HLA antigens from the serum onto their surfaces. It is not clear if all or only a few antigens are thus adsorbed. If antigens are adsorbed differentially, this could affect the perceived structure of the MHC in species where red blood cells are the main source of target cell.

Variability in the expression of HLA antigens on platelets has been reported (Svejgaard et al., 1971b). The significance of the observation is unclear. It does however suggest that in attempting to define class 1 antigens, platelets should not be the sole source of target cells.

b) Antigen expression in different tissues

Care has to be taken in interpreting results on the expression of class 1 in different tissues. There are several possible pitfalls. Firstly, different methods have been used by different authors. Different tests will have different sensitivity. Thus, one test may show antigens to be present at a low concentration. Another type of test may suggest that antigens are absent altogether. Secondly, many results could be due to tissue contamination with platelets and red and white blood cells. Thirdly, tissues are composed of many cell types. Different cell types could express different amounts of class 1 antigens.

I have broken down the discussion on different tissues into three parts; they are, tissues which express readily detectable antigen, tissues/

tissues which express little or no antigen and finally, tissues on which the expression of antigens is doubtful.

i) Tissues which express readily detectable antigen

The tissues which have been shown to possess easily detected antigen are in man, spleen, lung, liver, intestine, kidney and heart (Albert and Götze, 1977); in rabbits, spleen, lung, liver, kidney and heart (myocardium) (Matej, 1970a); in rats, liver, kidney and heart (Hausmann and Palm, 1973), in chickens, spleen, liver and bone marrow (Benda, 1971; Waters and Bennet, 1971) and in mice, spleen, lung, liver, gut, kidney, heart, lymph node, thymus, adrenal gland and salivary gland (Klein, 1975). In guinea pigs evidence for the existence of transplantation antigens on spleen, lungs, liver and kidneys has been obtained by Kahan (1967) and by Kahan and Reisfield (1967).

ii) Tissues which express little or no antigen

The tissues are, in man, brain and fat (Berah, 1970); in rabbits, brain (Matej, 1970a); in chickens, brain and muscle (Klein, 1975). In contrast, in rats, brain was observed to possess similar amounts of antigen to kidney and heart tissue (Hausmann and Palm, 1973).

iii) Tissues on which the expression of antigen is doubtful

In mice, trophoblast and nervous tissue fall into this category (Klein, 1975). In contrast in man, trophoblast (Goodfellow et al., 1976; Faulk and Temple, 1976) appears to have a low concentration of HLA antigens. The presence of HLA antigens on nervous tissue is not known.

In summary, a comparison of the known tissue distribution of class 1 MHC antigens, supports the concept that the loci in the different species belong to homologous systems. Although, there are significant differences between species. Class 1 antigens occur on nearly all tissues investigated. There are however quantitative differences in antigen expression between tissues. It is not known if the differences in quantitative expression are due to differences on all cells of a tissue or alternatively to antigen presence on some cells of a tissue and antigen absence on other cell types.

5. Biochemistry

The discussion on biochemistry has two purposes, firstly to compare the data obtained in different species for evidence of homology and secondly to discuss the likely structure of the class 1 molecules. I have divided this section into four parts.

a) Molecular weight and structure

In man class 1 antigens are apparently dimers. One molecule has a molecular weight of 44,000 daltons. The other molecule has a molecular weight of 12,000 daltons and it is B-2 microglobulin. This structure holds true for antigens of the HLA A, B and C loci (reviewed by Albert and Götze, 1977). Interestingly, B-2 microglobulin is coded for by a locus which is not linked to HLA (Goodfellow et al., 1974, 1975). Similar results have been reported for mice (Klein, 1979b), guinea pigs (Finkelman et al., 1975) and chickens (Pink et al., 1975; Ziegler and Pink, 1975). The smaller subunit has not yet been identified as B-2 microglobulin in either chicken/

chicken or guinea pigs. In the rat, respective molecular weights are 37,000 and 11,000 daltons (Katagiri et al., 1975a,b). The smaller molecule was shown to be B-2 microglobulin by testing with human and mouse antisera.

In pigs, Chardon et al. (1978) have determined the molecular weight of the two components to be 30,000 and 12,000 daltons respectively. The smaller component was shown to be B-2 microglobulin. The comparatively low molecular weight of the heavy polypeptide may be due to the use of papain in the solubilization procedure. Springer and Strominger (1976) have shown that papain causes degradation of HLA molecules and that this degradation results in reduced estimates of molecular weight.

b) Presence of carbohydrate

The 44,000 dalton polypeptide has been shown to be a glycoprotein in both man (Terhorst et al., 1976) and mouse (Klein, 1975). The molecular weight of the carbohydrate component has been estimated at 3,000 daltons in man (Terhorst et al., 1976) and 3,300 daltons in mouse (Nathenson and Muramatsu, 1971).

c) Molecular basis of alloantigenicity

Variation in amino acid sequence are believed to account for the antigenicity of the class 1 alloantigens in both man (Parham et al., 1977; Snary et al., 1977) and mice (Klein, 1975).

d) Amino acid sequencing

Complete amino acid sequences are not yet available for any class 1 molecules. Nonetheless, preliminary data indicate:-

i)/

i) Sequence homology between different class 1 loci of the same species. This has been shown for H-2D and H-2K in mice (Nathenson and Cullen, 1974) and for HLA-A and HLA-B in man (reviewed by Barnstable et al., 1978). Several authors have argued that the different class 1 loci arose by gene duplication (e.g. Klein, 1979b). The homology between the different class 1 gene products supports this argument. Ohno (1970) has argued that gene duplication is a method of ensuring permanent variation in the genome. This hypothesis would explain the existence of several class 1 loci in different species.

ii) Sequence homology between different species. Klein (1979b) presents NH_2-terminal amino acid sequences of class 1 molecules from mouse, man, rat, guinea pig and chicken. Kimball et al. (1979) have also claimed sequence homology with a rabbit class 1 molecule. There is impressive similarity between species. The similarity suggests significant homology between different species.

In summary, biochemical analysis of class 1 antigens suggest strong similarities between different species. The class 1 antigens appear to be dimers. The heavy chain is a glycoprotein, with a molecular weight of 40-45,000 daltons. The light chain is B-2 microglobulin and has a molecular weight of 11-12,000 daltons. The difference between gene products at any one locus is probably due to amino acid sequence changes in the heavy chain.

6. Genetic Polymorphism

One of the interesting features of the class 1 MHC genes is the large number of alleles that are coded for by each locus. Thus in man, there are at least nineteen alleles at the HLA-A locus, at least twenty-eight alleles at the HLA-B locus and at least seven alleles at/

at the HLA-C locus (Joysey and Wolf, 1978). In all species so far studied there appears to be a high degree of polymorphism amongst class 1 loci. Shonnard et al. (1976) found that specific allo-antisera to the eight known AgB groups of the rat also reacted with a high proportion of wild rats. In total, thirty-seven wild rats were tested, twenty-one possessed one AgB group and fourteen possessed two AgB groups. From this, they claimed that there was restricted genetic polymorphism at the rat MHC. However, it is not clear if the AgB groups define alleles or antigenic determinants common to several alleles. Klein (1970, 1972) has found that public antigens defined in inbred strains of mice are quite common in wild mice although private antigens are rare. This result suggests that wild mice also show extensive polymorphism of class 1 loci in mice.

High levels of polymorphism can be explained in three ways. The different alleles may be without selective advantage (or disadvantage) i.e. the alleles are selectively neutral. Alternatively, the polymorphism could be due to the effects of natural selection, e.g. selection may favour heterozygotes. It is also possible that selection could be acting on a gene in linkage disequilibrium with the class 1 loci. This selection would maintain polymorphism of the class 1 loci even though they themselves were selectively neutral. (The extent of linkage disequilibrium in the MHC is discussed later.)

At this moment, it is felt that the MHC polymorphism is maintained by selection but this has not been proved. Several authors (e.g. Klein, 1979b) have argued that as no system is as polymorphic as the MHC,/

MHC, to accept the hypothesis that the polymorphism is without selective advantage, must mean that no other neutral locus exists. However, other highly polymorphic genes do exist e.g. Xanthanine dehydrogenase in Drosophila melanogaster (Johnson, 1978 - personal communication) and the B blood group in cattle (Bouw and Fiorentini, 1970). Moreover, the methods used to detect polymorphisms may not detect all alleles. This has been argued for electrophoresis (Lewontin, 1974) and is almost certainly true for serological methods.

W. F. Bodmer (1973) has suggested that polymorphic loci might be composed of a string of genes located on the DNA. Each string would have a regulator gene which controlled the synthesis of just one gene in the gene-string. There is no evidence for or against this hypothesis at present. However, certain syngeneic tumours in inbred mice express foreign antigens, with most antigens being tumour specific. Occasionally, however tumours express antigens which are apparently identical to specificities of the H-2 complex, but the specificities are not possessed by the other tissues of the host. The expression of alien histocompatibility antigens on tumour cells has been recently reviewed by Parmiani et al. (1979). If Bodmer's theory is correct, then the existence of alien histocompatibility antigens could be explained by gene derepression.

7. The Detection of Interspecies Homology by Serological Methods

Several authors have tested the cells of one species with alloantisera produced in another species. Thus, rabbit alloimmune sera have been shown/

shown to be selectively cytotoxic for human lymphocytes (Albert et al., 1969) and also for murine cells (Abeyounis and Milgrom, 1969). Iha et al. (1973) used cattle alloantisera on human lymphocytes and found significant associations between cattle antisera and the known HLA antigens on the human cells. Additionally, mouse antisera directed against known H-2 specificities have been shown to detect products of the HLA system (Ivašková et al., 1972, 1975, 1976; Pellegrino et al., 1974; Iványi et al., 1976, 1977). Absorption experiments suggested that antibodies against mouse public antigens were responsible. Further, the relationship between the class 1 antigens of man and the other primates has also received considerable attention. Balner (1977), in reviewing the literature on the MHC antigens of primate species, concluded that man and chimpanzees shared antigens. However, with the exception of Bw4 (4a) and Bw6 (4b) the known human specificities did not appear to be present on either gorillas or orang-utans. Bw4 (4a) and Bw6 (4b) were present on cells from gorillas, orang-utans, chimpanzees, stumptail macaques and rhesus monkeys. However, Balner (1977), emphasised that the results obtained should be treated with caution. There are many pitfalls in interspecies typing. In many cases absorption have not been carried out. Even in chimpanzees and humans where the existence of shared antigens is well established, Dorf et al. (1972) using absorption and elution studies were able to show distinct differences between HLA-A11 and its chimpanzee counterpart ChLA-W108.

C. Class 2 Loci

As defined by Klein (1977), class 2 loci include genes coding for Ia antigens, genes coding for lymphocyte activating determinants and immune response genes. Each category is discussed separately.

1. Ia Antigens

Ia antigens were first discovered in the mouse (David et al., 1973a; Hauptfeld et al., 1973). They were called Ia antigens (Shreffler et al., 1974) as they were coded for by genes in the mouse I region (I region associated).

Currently, at least three loci are known to code for Ia antigens in the mouse. Ia-1 maps in the I-A subregion, while Ia-2 maps in the I-E/C subregion. The third locus, which appears to produce an antigen present on suppressor T cells only, maps in the I-J subregion (Murphy et al., 1976; Tada et al., 1976).

Ia antigens have also been found in the human. They were the major topic of the seventh international histocompatibility workshop (Bodmer et al., 1978). Currently, eight antigens are known. They are all thought to be coded for by one locus. The locus is called HLA-DR. The coding for the antigens are called DRw1, DRw2, DRw3, DRw4, DRw5, DRw6, DRw7 and W1A8. As with the class 1 antigens, the 'w' refers to a workshop or provisional specificity.

Seven Ia antigens have been found in guinea pigs (Schwartz et al., 1976, 1978a). They are called Ia.1, Ia.2, Ia.3, Ia.4, Ia.5, Ia.6 and Ia.7. There is no evidence to indicate that more than one locus is involved.

In rhesus monkeys, eleven "Ia-like" specificities have been postulated. Two genes are thought to be involved in controlling eight of the eleven antigens (Balner et al., 1976; Roger et al., 1976).

Evidence for Ia antigens in rats has been presented by Butcher and Howard (1977), by Shinohara et al. (1977) and by Soulillou et al. (1976). The number of specificities present in rat strains is not known. Although, Shinohara et al. (1978) and Shinohara and Sachs (1979) have used sequential precipitation with mouse anti-Ia alloantisera and rat anti-rat Ia sera to suggest that at least two loci are involved.

In pigs, evidence for the existence of Ia antigens was first presented by Vaiman et al. (1975). Lunney and Sachs (1979) have developed three inbred strains of miniature pigs. They have so far defined four Ia antigens Ia.1, Ia.2, Ia.3 and Ia.4. One inbred line carried Ia.3 and Ia.4. In sequential precipitation Ia.3 and Ia.4 do not precipitate together. Lunney and Sachs (1979) have argued that this observation means that at least two loci must code for Ia antigens in pigs.

Evidence for the existence of Ia antigens in chickens has been presented by Ewart and Cooper (1978).

Alloantisera which react with only a proportion of peripheral blood lymphocytes have also been found in cattle (Cwik et al., 1979; Newman, 1979 - personal communication).

It is generally believed that the Ia antigens described in different species belong to homologous loci. The evidence for this comes from four sources.

a. Firstly, the tissue distribution of Ia antigens has been found to be very similar in different species. By definition, Ia antigens are linked to the MHC and are only found on a proportion of lymphocytes. In the mouse, Ia antigens occur mainly on B lymphocytes (Sachs and Cone, 1973; Hämmerling et al., 1974) but they are also expressed on T lymphocytes (Frelinger et al., 1974; Götze, 1975) and weakly expressed on thymocytes (David et al., 1973a). A similar situation has been found in rats. T lymphocytes and thymocytes bind 2% and 10% respectively of the antibody bound by peripheral B cells (Mason and Gallico, 1978). Similarly in guinea pigs, B cells contain considerably larger amounts of antigen than T cells (Shevach et al., 1973). In pigs, Vaiman (1979) has claimed that Ia antigens are present on B cells and also on T cell subclasses. Evidence of this has not yet been published. Ia antigens exist predominately on B lymphocytes in humans (reviewed by J. G. Bodmer, 1978) but they have also been found on some T cells (Fu et al., 1978; Metzgar et al., 1979). Ia antigens have been found only on B lymphocytes in chickens (Ewart and Cooper, 1978) and rhesus monkeys (Balner and van Vreesjwijk, 1975) but their presence on a low proportion of T cells or at a low concentration on T cells has not been excluded.

The distribution of Ia antigens on other non-lymphocytic cell types has been studied in men, mice, rats, guinea pigs, pigs and chickens. In man Ia antigens are present on monocytes (Bodmer et al., 1975; Stasny, 1978) and on epidermal Langerhans cells (Stingl et al., 1978b). In mice Ia antigens are present on macrophages, fetal liver cells, bone marrow cells, sperm and epidermal Langerhans cells (Delovitch and McDevitt, 1975; Hämmerling et al., 1975; Rowden et al., 1978). However,/

However, they do not occur on erythrocytes, fibroblasts, muscle, brain, kidney or liver cells (Hämmerling, 1976). In rats, Mason and Gallico (1978) have shown that Ia antigens are absent from erythrocytes and only present on kidney cells in very small amounts. In guinea pigs, Stingl et al. (1978a) have shown that Ia antigens occur on epidermal Langerhans cells. While in pigs, Ia antigens are found on sperm (Vaiman et al., 1978a) and epidermal cells (Vaiman, 1979). In chickens, Ia antigens have been found on cells of the monocyte-macrophage series (Ewart and Cooper, 1978).

b. The biochemical characterisation of Ia antigens lends further support to the concept of homology. Biochemical studies in mice (Cullen et al., 1976), man (Allison et al., 1978), rats (Blankenhorn et al., 1978), pigs (Lunney and Sachs, 1978) and guinea pigs (Schwartz et al., 1978b; Waxdal et al., 1978) have all demonstrated that Ia antigens are composed of two separate molecules. The larger (A) chain has a molecular weight in the range 31,000 to 37,000 daltons. The smaller (B) chain has a molecular weight of 25,000 to 29,000 daltons.

Amino acid sequences also suggest similarity. Thus, the NH_2-terminal amino acid sequence of the A chain of Ia-2 in the mouse shows significant homology with P34 (P34 is an A chain of a human Ia-like product). Also the B chain of Ia-1 and guinea pig B chain both show homology with human P29 (a B chain) (Cook et al., 1978). Allison et al. (1978) have also reported that human DR antigen shows NH_2-terminal amino acid sequence homology with a mouse Ia-2 antigen.

c. The relationship of Ia antigens to genes responsible for mixed lymphocyte culture also suggests species homology. The genes responsible for Ia antigens are often thought to be responsible for stimulation in MLC (e.g. Albert and Götze, 1977). Although this has been disputed (e.g. Bradley and Festenstein, 1978).

The evidence for gene identity comes from three sources:-

i) anti-Ia sera inhibit mixed lymphocyte reactions in mice (Meo et al., 1975a). Suggestive evidence has also been obtained in the human (Revillard et al., 1972; van Leeuwen et al., 1973).

ii) genes controlling specificities defined by MLC and anti-Ia sera map very close together and may even be the same position. This is true for both man (Bodmer et al., 1978) and rhesus monkeys (van Es and Balner, 1978). Similarly, in mice, lymphocytes activating determinants (lads) map in the same areas of H-2 as Ia antigens (i.e. in the I-A and I-E/C subregions).

iii) the tissue distribution of Ia antigens and lads is very similar. Thus, in man, the following cells have caused stimulation of lymphocytes; B and T lymphocytes (Lohrman et al., 1974), monocytes (Marshall et al., 1966; Hirschberg et al., 1976), epithelial cells (Cochrum et al., 1971; Levis and Miller, 1972; Hirschberg and Thorsby, 1975), endothelial cells (Hirschberg et al., 1974) and sperm (Halim and Festenstein, 1975; Festenstein and Halim, 1976). In contrast granulocytes (Johnson et al., 1971; Ragab and Cowan, 1973), platelets (Albert and Götze, 1977) and fibroblasts (Schellekens and Eijsvoogel, 1970) do not cause lymphocyte proliferation. Kidney cells also possess lad and Ia antigens. In contrast, liver cells are lad positive but Ia negative (Bradley and Festenstein, 1978).

d. The final piece of evidence in favour of species homology is the observation that alloimmune sera raised within a species have been shown to react with cells of another species.

Thus, anti-Ia sera raised in mice were shown to be cytotoxic for a proportion of peripheral blood lymphocytes drawn from humans and rats (Iványi, 1977 - personal communication; Shinohara et al., 1978). As the specificity of the target antigens was not known, the results may not be due to similarity of Ia antigens in different species. However, mice alloimmune sera have been used against radiolabelled lysates of rat lymphocytes (Shinohara and Sachs, 1979) and hamster lymphocytes (Phillips et al., 1979). In both cases, biochemical properties of the target antigens suggested homology with mouse Ia antigens.

In conclusion, a study of Ia-like antigens in different species supports the assumption of similarity between species and suggests that every MHC will contain genes coding for antigens of restricted tissue distribution.

2. Cellular Typing

When lymphocytes from unrelated donors are cultured together in suitable conditions, they usually divide and proliferate rapidly. The phenomenon is called MLR (mixed lymphocyte reactivity) or MLC (mixed lymphocyte culture). When cells from both donors are free to divide, the test is called a two-way test. Usually, one set of cells (stimulators) are inactivated with x-irradiation or mitomycin C to prevent growth. The MLC test in this case only measures the response/

response of cells from one donor (responder). This is called a one-way test. Unrelated individuals rarely fail to stimulate each other. The assumption is that the responding cells proliferate because the stimulating cells express foreign cell surface determinants. When the stimulating and responding cells come from one individual there is little or no response. Failure to respond is interpreted as genetic identity for the stimulating cell surface determinants.

Bach et al. (1969) have shown that mixed lymphocyte reactivity in man is controlled by the HLA system (i.e. the stimulating determinants are coded for by genes within HLA). The occurrence of recombination in several families was used to demonstrate that the genes coding for class 1 antigens were not responsible for the majority of variation in MLR (reviewed by Bach and van Rood, 1976a,b,c).

In nine species, the same genetic system that controls the expression of class 1 antigens has also been shown to control MLR. The nine species are mice (Dutton, 1966), rhesus monkeys (Appleman and Balner, 1972), chimpanzees (Siegler et al., 1974), dogs (Grosse-Wilde et al., 1973), pigs (Vaiman et al., 1970a), guinea pigs (Geczy and de Weck, 1976), rabbits (Tissot and Cohen, 1974), rats (reviewed by Günther and Stark, 1977) and chickens (Miggiano et al., 1974). In mice, an additional locus, the M locus, has also been shown to cause lymphocyte proliferation (Festenstein et al., 1972). However, the response to the M locus has different kinetics than the response to the MLR locus. The two effects can therefore be readily distinguished.

In hamsters, lymphocyte antigens are not yet described. However, MLR segregated with skin graft rejection and graft versus host reactivity (Duncan and Streilein, 1976). In the clawed toad, alloantisera have been raised by skin grafting and erythrocyte injections. The antisera, once purified by absorption, detect specificities which segregate with MLR (Du Pasquier et al., 1975). In cattle, the link with lymphocyte antigens has been suspected but has not yet been proved.

The control of MLR in different species can be seen to be very similar. Further evidence for homology of the different systems comes from the work of Martinis and Bach (1977). They used a modification of the MLC test, the primed lymphocyte typing test (PLT). Stimulated lymphocytes in a MLC, if allowed to proliferate, revert to small lymphocytes by ten days. If the lymphocytes are then restimulated by the original cell or by another cell which carries the same determinants as the first cell, proliferation after the second stimulation is much more rapid than it was originally. This is known as a secondary response. Restimulation with a cell which does not share determinants gives a primary response. The secondary response can be distinguished from the primary by testing the response 24 hours after the second stimulation. At this time, a secondary response would be underway, while a primary response would hardly have started. Martinis and Bach took human lymphocytes and primed them with a pool of human lymphocytes. This was to prime the responding lymphocytes against all determinants (except those which they themselves possessed). The primed human lymphocytes were then stimulated with lymphocytes from rhesus monkeys, dogs, cattle or mice. Restimulation with human cells served/

served as a control. Normal (unprimed) human lymphocytes were also tested in an MLC test with lymphocytes from animals of the same four species. Xenogeneic primary MLC test invariably caused cell proliferation. PLT tests caused proliferation with 10/10 rhesus monkeys, 13/14 dogs and 8/30 cattle. No proliferation was observed in any of 34 mice tested. The mice came from seventeen strains. Eleven independent and six recombinant H-2 haplotypes were represented. The PLT stimulation was taken as evidence of shared determinants. The differences in MLC and PLT tests were interpreted by the authors to mean that additional (non-MHC) determinants acted in xenogeneic MLC.

In all species which have been examined for allogeneic MLC, there is a relatively low proportion of unrelated individuals which fail to cause stimulation. This is taken as evidence of extensive polymorphism. The observed polymorphism could be due to a single gene with a high number of alleles. Alternatively a few genes each with a relatively small number of loci could explain the polymorphism, if incompatibility at one locus was sufficient to cause stimulation. The genetic control of MLR is far from clear. At least two genes are reported to be necessary in mice (reviewed by Klein, 1975), rats (Günther and Stark, 1978),and cattle (Usinger et al., 1977). However, there is considerable variation in MLC and PLT tests and precise genetic control is difficult to establish. The available data provides no evidence for dissimilarity of genetic control of MLR between species.

Further evidence for the similarity of the MHC in different species comes from the phenomenon of cell mediated lympholysis (CML). A test for CML was developed by Lightbody et al. (1971) as an extension of the MLC test. A CML test measures the killing of target cells by effector/

effector cells. The effector cells are lymphocytes grown up in MLC. The target cells are lymphocytes cultured with a suitable mitogen (e.g. PHA). The PHA transformed lymphoblasts are mixed with the MLC stimulated effector cells. The test measures the killing of the target cells. In both humans (Goulmy et al., 1976; Sondel and Bach, 1976) and mice (Alter et al., 1973) the target antigens are part of or very closely linked to the MHC.

In summary, cellular typing experiments have also demonstrated a similarity between the MHC of different species.

3. Immune Response Genes

The immune response to small doses of certain selected antigens appears to be controlled by a single autosomal dominant gene (Kantor et al., 1963). Immune response (Ir) genes are responsible for:-

a) quantitative variations in the amount of antibody synthesised. This can apply to all or only to a few classes of immunoglobulin,

or

b) qualitative variations. No antibody is synthesised in animals lacking the relevant gene.

Ir genes have been shown to be linked to the MHC in mice (reviewed by Benacerraf and Katz, 1975a), guinea pigs (Ellman et al., 1970), chickens (Günther et al., 1974; Balcarová et al., 1974; Benedict et al., 1977; Koch and Simonsen, 1977), rhesus monkeys (Balner, 1973; Balner et al., 1973a; Dorf et al., 1974, 1975), pigs (Vaiman, 1978c) and rats (reviewed by Günther and Stark, 1977). Preliminary evidence has also been obtained in man (Levine et al., 1972) but this has been disputed

(Bias/

(Bias and Marsh, 1975). Ir genes have been looked for in dogs but the results are inconclusive (Vriesendorp et al., 1977).

The number of different loci involved and the mode of action of Ir genes has been reviewed by Benacerraf (1972, 1974, 1977a,b), Benacerraf and Katz (1975b) and Benacerraf and Germain (1978).

D. Class 3 loci

In several species certain complement components have been shown to be linked to the MHC. Klein (1977) defines the MHC-linked complement genes as class 3 loci. Thus in humans, Fu et al. (1974) have shown that a gene responsible for C2 deficiency is linked to HLA. Linkage between C2 and the MHC has also been reported for guinea pigs (Bitter-Suermann et al., 1978). Another complement component C4 is linked to the MHC in man (Rittner et al., 1975) and guinea pigs (Shevach et al., 1976). In mice, the levels of a serum protein Ss and an allotypic variant slp are controlled by the H-2 complex. Ss-slp is probably the murine C4 component. (Curman et al., 1975; Lachman et al., 1975; Meo et al., 1975b). Properdin factor B also appears to be closely linked to the MHC. Linkage has been shown for humans (Allen, 1974), rhesus monkeys (Ziegler et al., 1975), guinea pigs (Kronke et al., 1977) and mice (Rubinstein and Day, 1978).

Genes responsible for the general levels of activity of haemolytic complement also appear to be linked to the MHC in chickens (Chanh et al., 1976), hamsters (Duncan et al., 1977) and pigs (Vaiman et al., 1978b). As complement functions as a cascade process, it is possible that the decreased levels of activity observed in some animals is due to an alteration in just the activity of one (or a few) component(s). This is the explanation favoured by Chanh et al. (1976).

There are two examples of dissimilarity between the MHC of different species with respect to linkage of complement genes. a) Goldman and Goldman (1975) found that the haemolytic activity of C1 is associated with Ss level in congenic strains of mice. However, Mittal et al. (1976) found no linkage between HLA and C1 in humans. Also, Day et al. (1975) found no linkage between C1r deficiency and HLA. The contrasting observations in mice and humans may reflect a genuine difference between H-2 and HLA. However two other explanations are possible. Firstly, the association between C1 and H-2 may not be real. The evidence presented by Goldman and Goldman is not conclusive. Not all associations between C1 and Ss levels were significant. The observed haemolytic levels could be a secondary effect of environmental or possibly other genetic influences. Secondly, Goldman and Goldman looked at the levels of complement activity while Mittal et al. and Day et al. used the absence of the gene as a marker. It is possible that activity might be controlled by a regulatory gene, while the C1 deficiency could be due to a defect in a structural gene. The structural and regulatory genes need not be linked. b) The second example of dissimilarity between species concerns C3. C3 is not linked to HLA (Gedde-Dahl et al., 1974). Yet C3 does appear to be linked to H-2 in mice (Ferreira and Nussenweig, 1975; Ferreira and Nussenweig, 1976; da Silva et al., 1978). However, C3 is not closely linked to H-2 (recombination frequency = 10%). C3 therefore lies outside the H-2 complex. Linkages of this order are not necessarily maintained in evolution. Alternatively several genes may be responsible for the observed variations in C3 levels. The studies in mice and men could be looking at the effect of different genes.

The linkage relationships to HLA of complement components C6, C7 and C8 have also been investigated. Hobart et al. (1977) and Olving et al. (1977) found no linkage between C6 polymorphism and HLA. While Mittal et al. (1976) found no association between C6 polymorphism and HLA. However Raun et al. (1976) did find an association between C6 deficiency (50% normal C6 levels) and HLA. Three out of four propositi had HLA-Aw 24. C7 does not appear to be linked to HLA (Prochazka et al., 1976; Delâge et al., 1977). Hobart et al. (1978) have demonstrated that C6 and C7 are linked to each other. Merrit et al. (1976) reported linkage of a C8 deficiency to HLA. Although this has not been confirmed by other studies (Day et al., 1976; Jasin, 1977). Also a polymorphism for C8 has been described. It too does not appear to be linked to HLA (Alper, 1978).

Finally, in mice, there is a locus within, or very closely linked to, the MHC which controls the ontogenic expression of the C3b receptor (Gelfand et al., 1974; Arnaiz-Villena et al., 1975). Curry et al. (1976) using man-mouse somatic hybrid cells found linkage between the MHC in man and receptors for C3b and C3d.

The linkage of complement components to the MHC has been reviewed more fully by Hobart and Lachmann (1976), Hobart (1977) and Lachmann and Hobart (1978).

E. Genetics of the MHC

1. Genetic Organisation

I have restricted the discussion on the genetic organisation of the MHC to those species where the position of at least three loci is known/

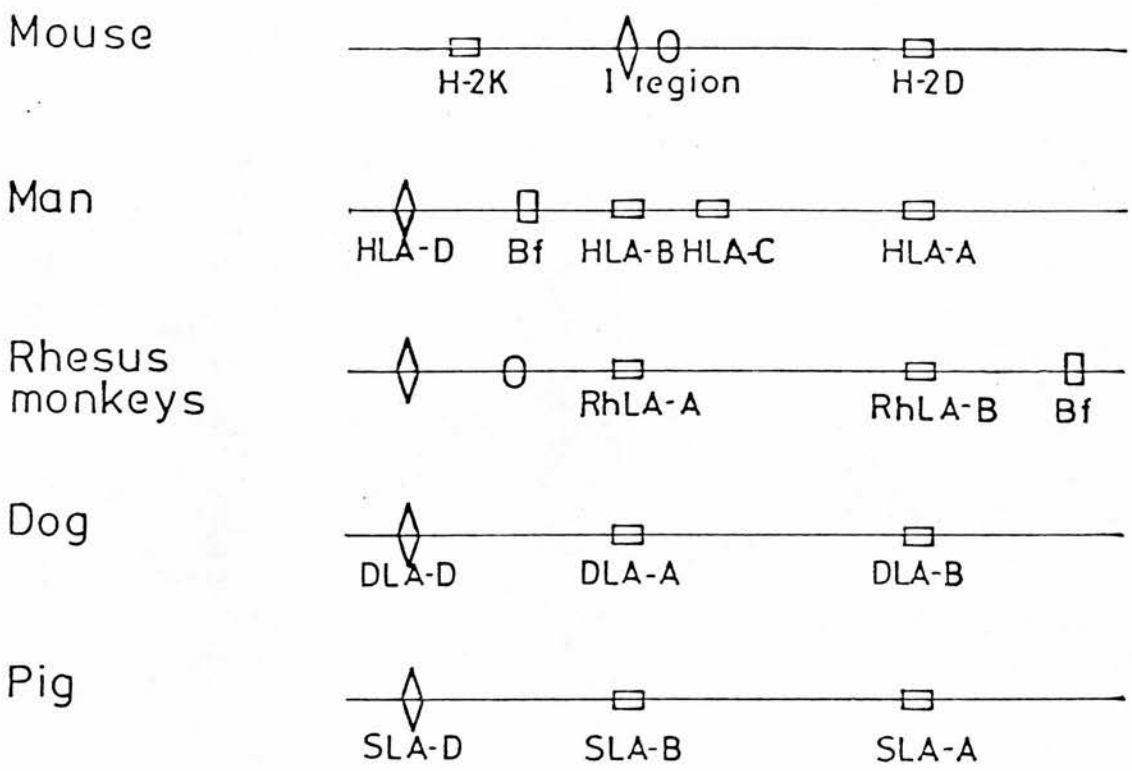
known with certainty. Five species meet this criterion. They are mice (Klein, 1979b), man (Albert and Götze, 1977), pigs (Vaiman, 1979), dogs (Vriesendorp et al., 1977) and rhesus monkeys (Balner, 1977). The genetic organisation of the MHC in these species is given in figure 2. Loci which have only been mapped in one species have been omitted (e.g. C4 in the mouse). Also, Ia antigen genes have not been shown as they may be identical to lad genes.

In the mouse, genes controlling Ia antigens, immune responsiveness and lymphocyte activating determinants have been mapped between H-2K and the Ss-Slp locus (which lies between H-2D and H-2K). This region is called the I region in mice. The region has been subdivided into four parts, I-A, I-B, I-J and I-E/C. Originally I-E and I-C were thought to be separable by recombination, but this is no longer the case. Several authors have suggested that Ia, lad and Ir genes will map together to form an I-region in all species (e.g. Adams et al., 1979). Immune response and lad genes have been mapped together in rhesus monkeys, but the situation in other species is not yet known.

In the mouse, the I region maps between the class 1 loci (Klein, 1975). In contrast, in man, rhesus monkeys, dogs and pigs, the putative I-region maps outside the class 1 loci. Hauptfeld and Klein, (1976) have shown that the I-region also occurs between H-2K and H-2D in wild mice. Thus, the I-region position is not an artefact of inbred strains. Gill and Kunz (1976) looked at the position of an Ir gene, an lad gene and two class 1 loci in inbred strains of rats. As the lad locus and, possibly the Ir gene also, mapped outside the two class 1 loci, they claimed that even the rat MHC/

Figure 2

The Genetic Organisation of the MHC in Different Species



Key

- ▭ Class 1 locus
- ◊ Locus controlling lymphocyte activating determinant
- O Immune response gene
- ▯ Properdin factor B

MHC was more similar to the human than to the mouse model. It is not clear why the genetic organisation of the MHC should be different in mice. Also, the significance of this difference is unclear.

2. Haplotype Nomenclature

Workers defining the MHC in several species have been able to make use of pre-existing or specially-created inbred strains. This is true of mice, rats, chickens and to a lesser extent, guinea pigs and hamsters. The use of inbred strains in mice and rats has created a need to describe haplotypes. The combination of genes on any given chromosome is called a haplotype. Outbred animals possess two haplotypes which are usually different. Inbred animals possess two identical haplotypes. In chickens, haplotypes have not been designated. This is partly due to the presence of large outbred flocks and partly due to the fact that fewer genes are described in the MHC. Similarly, haplotypes in guinea pigs and hamsters are not designated. Again, this is probably partly due to the fact that fewer genes have been described and also, perhaps, to the fact that fewer inbred strains are available.

In the mouse, the H-2 haplotypes are each designated by small letters and numbers in a superscript position, e.g. H-2^{a} refers to the 'a' haplotype. Haplotypes derived from wild mice are given the superscripts w1, w2, w3, etc. (Klein et al., 1974). Symbols H-2^{c}, H-2^{e}, H-2^{1}, H-2^{n} and H-2^{w} are not used (Klein, 1975).

There are more haplotypes than letters available. The extra haplotypes are designated by a two letter superscript, e.g. H-2^{ap}. H-2^{ap} has no necessary connection with H-2^{a}. The second letter of the superscript/

superscript must come from the last eleven letters of the alphabet (p to z). Recombinant haplotypes are designated by the original superscript followed by an Arabic numeral e.g. H-2^{g1}, H-2^{g2}. The numeral distinguishes members of the same recombinant family. Mutant haplotypes are designated by the addition of the letter 'm' to the previously assigned haplotype symbol followed by an Arabic numeral e.g. H-$2^{bm}1$. Variant haplotypes are similarly named except that a 'v' is used instead of an m. Full details of the rules and the changes necessary in the designation of mutant and variant haplotypes have been given by Kohn et al. (1978). In the rat, the major histocompatibility system has received a variety of names (Palm, 1970). These names have included Ag-B (Elkins and Palm, 1966), RtH-1 and H-1 (Štark and Křen, 1967) and the R-1 system (Bogden and Aptekman, 1960). The system nomenclature is currently under review. The Ag-B haplotypes are designated by numbers (Ag-B^1, Ag-B^2, etc.). The H-1 haplotypes are designated by letters (H-1^a, H-1^b, etc.). For a discussion on the relationships between the two nomenclatures see Günther and Štark (1977).

3. Linkage Relationships

A study of the linkage relationships of the MHC lends further support to the concept of homology between species.

Thus, red cell glyoxylase (GLO) is linked to the MHC in man (Bender and Grzeschik, 1976; Kömpf et al., 1976; Mayr, 1976; Weitkamp, 1976) and mice (Meo et al., 1977; Leinwand et al., 1978). Also the third locus controlling phosphoglucomutase enzymes (PGM_3) is linked to the MHC in man (Lamm et al., 1971; van Someren et al., 1974) and in dogs (Meera Khan et al., 1976).

Red blood cell antigens have been shown to be linked to the MHC in man, mice, pigs, rabbits and horses. In man, two red cell antigens were shown to be in very tight linkage with the HLA system - antigens Chido (Middleton et al., 1974) and Rodgers (Giles et al., 1976). Subsequently, O'Neill et al. (1978) showed that Chido and Rodgers are antigenic determinants of the fourth complement component. In Mice, the H-2G region contains a locus coding for H-2.7 which is a specificity found predominantly on red blood cells. David (1979) has suggested that H-2.7 maps in the S region. The S region also controls the expression of murine C4. This suggests that H-2.7 may be analogous to Chido or Rodgers in the human. Red blood cell antigen loci have also been mapped outside the MHC. In mice, erythrocyte-antigen 2 (Ea-2) is linked to H-2 (Klein, 1975). In pigs, Hruban et al. (1976) have demonstrated that the blood groups J and C are linked to SLA. Tissot and Cohen (1974) have shown linkage of the He blood group and the rabbit MHC. Also Bailey et al. (1979) have demonstrated linkage between the A red blood cell system and antigens of the horse MHC. It is not clear to what extent the red cell systems of the different species are homologous. It may be that the red cell systems are not at all homologous and the observed linkage relationships are not due to interspecies similarity.

Similarity between the MHC of mice and guinea pigs with respect to the Tla region has also been claimed. The Tla region is well established in mice (reviewed by Flaherty, 1978). Briefly, at least nine series of polymorphic cell surface antigens have been located in this region. Six of these, Tla, Qa-1, Qa-2, Qa-3, H-31 and H-32 are discussed by Flaherty (1978). Two others, Qat-4 and

Qat-5/

Qat-5 were found by Hämmerling et al. (1979). A ninth antigen, Qed-1 which is defined by the use of cytotoxic T cells was found by Lindahl and Hausmann (1979). Antigen Qed-1 may be the same as antigen Qa-1. Two enzyme loci, phosphoglycerate kinase-2 and kidney catalase-2, also map in the Tla region. Biochemical analysis has been carried out on the TL and Qa-3 antigens. Vitteta et al. (1972) found a molecular weight of 45,000 daltons for the TL antigen. Additionally, this antigen has B-2 microglobulin as a sub-component (Anundi et al., 1975). Michaelson et al. (1977) found that the Qa-3 antigen also had a molecular weight of 45,000 daltons and was also associated with B-2 microglobulin. Initially, Michaelson et al., (1977) believed that they were studying the Qa-2 antigen. They have subsequently (Michaelson, 1978) stated that the antiserum used for precipitation contained Qa-2 and Qa-3 activity. They believe that the antigen analysed was Qa-3. The similarity in biochemical structure of TL and Qa-3 antigens with class 1 MHC antigens has led several authors to include the Tla complex as part of the murine MHC (e.g. Klein, 1979b). Schwartz et al. (1978a) claimed to have detected guinea pig homologues of the TL and Qa-3 antigens. (In the original article, Schwartz et al., claimed homology with Qa-2. In the light of the above this homology is obviously with Qa-3. I have amended the review accordingly). The procedure followed was sequential immunoprecipitation of guinea pig lymphoid lysates with an antiserum against guinea pig B-2 microglobulin. After precipitation of the class 1 loci, which also have B-2 microglobulin as a sub-component, three molecules reacted with the antiserum. Their molecular weights were 40,000 daltons, 36,000 daltons and 12,000 daltons. When the tests were repeated in the presence of a protease inhibitor identical results were obtained. This implies that/

that the three molecules were not breakdown products but different entities. Schwartz et al. assumed that the 12,000 daltons molecule was B-2 microglobulin and that the two larger molecules were precipitated because they were associated with B-2 microglobulin. There were then two possible explanations for their results:-

i) the three molecules formed a single complex. However, they claimed that when the tests were repeated in non-reducing conditions identical results were obtained. This implied that there were no disulphide bonds holding the trimer together.

ii) Alternatively, two different dimeric complexes were involved. One dimer was the 12,000 and 40,000 dalton molecules. The other was composed of the 36,000 and 12,000 dalton molecules.

As the 40,000 but not the 36,000 dalton molecule was found on lymph node cells, Schwartz et al. argued that the molecules were not associated in a trimer.

Studies with lentil lectin, which binds to carbohydrate showed that both complexes contained carbohydrate. Schwartz et al. then argued that, as B-2 microglobulin does not contain carbohydrate, then 40,000 and the 36,000 dalton molecules must both be glycoproteins.

Schwartz et al. used lymph node cells or thymocytes to look at the distribution of the two antigens. The 12,000 + 36,000 dalton dimer was found only on thymocytes. As this is similar to the mouse TL antigen, Schwartz et al. suggested that the two were homologues. They suggested that the difference of 9,000 daltons in molecular weights between the mouse TL antigens and the guinea pig antigen was/

was not significant. They also found no binding with antisera against mouse TL antigen (data not shown). They suggested that this was not unexpected and concluded that "our evidence is highly suggestive, but not conclusive, that this molecule is the guinea pig homologue of murine TL". The other molecule was found on both lymph node cells and thymocytes. The authors suggested that the molecule could therefore be a class 1 histocompatibility antigen or a homologue of Qa-3. As the guinea pig molecule was found in small quantities on lymphocytes, in contrast to class 1 histocompatibility antigens but in agreement with Qa-3 in the mouse, Schwartz et al. suggested that the guinea pig molecule was homologous to murine Qa-3. Unfortunately, Qa-3 is not found on murine thymocytes. Schwartz et al. suggested that this could reflect a discrepancy in radiolabelling techniques or alternatively a genuine difference between guinea pig and mouse antigens.

One important area of difference between the linkage relationship and MHC in different species concerns the t complex. The t complex contains several loci which code for spermatozoan and embryonic differentiation antigens. Mutant alleles can cause congenital defects and even death. Many of the mutant types interact with the T locus. The T locus has 3 types of alleles, + (wild-type) t, and a dominant mutant Brachyury (T). In the presence of wild type genes at both loci, there is normal tail length. T alone causes reduced tail length. T, in the presence of mutant t genes often results in total absence of the tail. The t complex has been reviewed by Bennett (1975). Although mutant t alleles are very common in wild mice, there is no evidence for the existence of the t complex in any other species. Amos and Ward (1975) have suggested that spina bifida/anencephaly might be analogous to t in the mouse. However, Bobrow et al. (1975) have disputed this.

Hammerberg and Klein (1975) have suggested that the t complex arose as a genetic accident. This implies that similar complexes will not exist in other species. Klein (1977) has suggested that although mutant t haplotypes are not to be expected in other species, nonetheless wild type alleles might be. Therefore, a search should be made for sperm and early embryo alloantigens. F9 antigen appears to fulfil this role (e.g. Hogan et al., 1977).

Many authors (e.g. Hammerberg and Klein, 1975) have stressed the role that the t complex might play in the evolution of H-2. Thus mutant t alleles can cause segregation distortion and suppress recombination. The H-2 complex might therefore be significantly different from the MHC of other species. This could explain the differences observed in the genetic organisation of the H-2 complex compared to other animal MHCs.

Other loci have been shown to be linked to the MHC. Thus, in man, von Someren et al. (1974) have used somatic hybrids to demonstrate that super oxide dismutase 2 (SOD-2) and malic enzyme 1 (ME-1) are on chromosome 6. Ruddle and Giblet (1975) have placed urinary pepsinogen 5 (Pg-5) on chromosome 6. The HLA system is also on chromosome 6 (Jongsma et al., 1973; Lamm et al., 1974). Therefore HLA, SOD-2, ME-1 and Pg-5 are all on the same chromosome. The gene order is not known.

In mice, in addition to the genes already mentioned, the following genes are also on chromosome 17:- Quaking (gk), hybrid sterility 1 (hst-1), histocompatibility 39 (H-39), Knobbly (Kb), retinal degradation - slow (rds), plasma protein (Plp), scopolamine modification of exploratory activity (Sco), acid phosphatane-liver (Apl), A-mannosidase (map-2), thin fur (thf) and immune response 5 (Ir-5) (Klein, 1979b).

4. Linkage disequilibrium

In a large population at equilibrium undergoing random mating, different gene products are expected to be randomly associated with all other gene products. There is no expectation that the alleles of different genes will occur together more often than predicted from their gene frequencies. This expectation applies to linked as well as unlinked genes. Recombination will maintain the random assortment of linked genes.

Where alleles at different loci occur together (or apart) on the same haplotype at a frequency greater than that expected by chance alone, this is known as linkage disequilibrium. Linkage disequilibrium can be caused by non-random mating or by small population sizes (Cavalli-Sforza and Bodmer, 1971). With linked genes, once non-random association has been created, it will exist for many generations even if the disturbing influence is removed. The approach to equilibrium is given by the formula -

$$\Delta D = (1-r)^t$$

where ΔD is the decrease in D. (D is a measure of the association of gametes, equal to $X_1X_4 - X_2X_3$; given two linked loci, each with two alleles A_1, A_2 and B_1 B_2, there are four types of gametes A_1B_1, A_1B_2, A_2B_1 and A_2B_2 that can exist. Their respective relative population frequencies are X_1, X_2, X_3, X_4 where $X_1 + X_2 + X_3 + X_4 = 1$).

r = recombination frequency

t = time in generations

Fisher (1930) pointed out that a non-random association between genes can also be maintained by selection. The size of the selective advantage will depend upon the size of r (the recombination frequency). If r is small, only a slight selective advantage is necessary (reviewed by Bodmer and Felsenstein, 1967).

Linkage disequilibrium between MHC loci has been found in man (Svejgaard et al., 1971a), dogs (van den Tweel et al., 1974; Grosse-Wilde et al., 1975), rhesus monkeys (Roger et al., 1976) and rats (Wagener et al., 1979). Linkage disequilibrium extends beyond the MHC complex in man. Both C2 deficiency (Fu et al., 1975; Hauptman et al., 1977) and 21-hydroxylase deficiency (Pollack et al., 1979) are in linkage disequilibrium in man. This problem has recently been considered by Hiller et al. (1978). In mice, Hammerberg and Klein (1975) have shown that H-2 and the t complex are in linkage disequilibrium.

In humans, the observed linkage disequilibrium are highest when specific antigens occur together (e.g. HLA-A1,B8; HLA-A3,B7). This suggests that the cause of disequilibria is for antigens to occur together (rather than apart). Negative deviations have been observed - but they are quite small and are thought to be secondary effects (Albert et al., 1975).

5. Number of Genes in the MHC

Bodmer (1972) has considered the number of genes in the human MHC. For a recombination frequency of about 0.8% between HLA-A and HLA-B and an average of 51 chiasmata per human meiosis, Bodmer estimated that the MHC occupies about 0.03% of the total human genome. Given approximately 3×10^9 nucletide pairs per human haploid complement and assuming an average of 450 nucleotide pairs per gene, then the human genome will contain 6.7×10^6 genes. As the MHC contains 0.03% of this, this gives an estimate of 2,000 genes in the HLA system. After allowing for the existence of non-functional DNA, Bodmer goes on to argue that the "number of cistrons in the HL-A region is at least in the hundreds". This value has been widely quoted. However, these figures should be treated with suspicion. A crucial factor is the number of genes in the human genome and this value is not known. Stern (1973) has argued that the value will lie between 10,000 and 100,000. If these figures are accepted, the number of genes lying between HLA-A and HLA-B is estimated to be between 3 and 30. Further not all genes are expected to be polymorphic. Non-polymorphic genes are unlikely to be detected serologically or to play a role in an allogenic system (e.g. transplantation, MLC).

It is now known that MHC genes lie outside HLA-A and HLA-B e.g. HLA-D. Even after allowing for the existence of these genes, it is possible that the total number of genes in the human MHC may be much fewer than commonly assumed. Given the extensive homology between the MHC's of different species, results obtained in man may be assumed to hold in other species. At this moment, however, the total number of genes in any MHC is not known and there are no reliable estimates of this value.

A similar argument to that used by Bodmer on the HLA system was used by Shreffler & Klein(1970) on the mouse H-2 system. Assuming all DNA to be functional, they estimated that there could be as many as 500 genes between H-2D and H-2K (this chromosomal segment includes the I region and the S region). In a subsequent publication it was stated by Shreffler and David (1975) that as only a small proportion of DNA was thought to be functional there could be as few as five genes between H-2D and H-2K.

F. Function of the MHC

The biological role of any of the products of the MHC is not known in any species. Several observations have suggested that the functions of the MHC products are likely to be the same in different species. These observations are discussed under the three headings, cellular collaboration, disease associations and transplantation. It is not my purpose to review all the data on MHC function but only that relevant to interspecies homology.

1. Cellular Collaboration

Recent work has suggested that MHC antigens play an important role in cellular interactions in the immune response. It appears in several systems that cells must carry the same MHC antigens before they can collaborate. This phenomeneon is known as MHC restriction.

Thus, co-operation between T and B lymphocytes requires MHC compatibility. This was first shown in mice (Kindred and Shreffler, 1972; Katz et al., 1973). Subsequently, Toivanen and Toivanen (1977) have shown that T and B lymphocytes must be compatible at the MHC for effective germinal centre generation in chickens.

Also, the efficient lysis of target cells by cytotoxic T cells requires that the target cell expresses the same class 1 molecules as the cytotoxic T cell. This phenomenon was first shown to apply to LCM-virus infected cells by Zinkernagel and Doherty (1974). Subsequently T cell killing has been shown to be MHC restricted in a variety of systems including minor transplantation antigens (Bevan, 1975; Simpson and Gordon, 1977). MHC restriction of T cell killing has also been shown in man (Goulmy et al., 1977; McMichael et al., 1977; Dickmeiss et al., 1977); rats (Marshak et al., 1977; Zinkernagel et al., 1977) and chickens (Wainberg et al., 1974; McBride, 1979). However MHC restriction has been looked for but not found in cattle (Rouse and Babiuk, 1977); dogs (Ho et al., 1978) and rabbits (Woan et al., 1978). It ought however to be added that in the dog, cattle and rabbit studies, the animals used were not typed for class 1 antigens. As unrelated animals were used, the expectation is that the animals will differ at the MHC, but cross-reactions or even genetic identity cannot be excluded.

Before T cells will proliferate in response to an antigen, macrophages must be present (reviewed by Golub, 1978). T cells apparently see the antigen in the context of the macrophage class 2 MHC antigens. Thus it has been shown in guinea pigs that the secondary T cell response is dependent upon the macrophages in the second stimulation carrying the same class 2 antigens as the macrophages in the first stimulation (reviewed by Thomas et al., 1977). A similar situation has also been shown in mice (Pierce et al., 1976).

Further information on the role of the MHC in the immune response is provided in the reviews by Munro and Waldmann (1978) and by Zinkernagel and Doherty (1979).

2. Disease Association

A large number of diseases have been shown to be associated with specific haplotypes and alleles of the HLA system (reviewed by Dausset and Svejgaard, 1975). In mice, susceptibility to several diseases has also been shown to be associated with MHC type, e.g. susceptibility to gross virus leukemia is controlled by a gene within the H-2 complex (Lilley, 1966). In chickens, susceptibility to Marek's disease (Hanson et al., 1967), Rous sarcoma (Schierrman et al., 1977; Collins et al., 1977) and to spontaneous autoimmune thyroiditis (Bacon et al., 1973) is linked to the MHC. In rats, susceptibility to three artificially induced diseases is linked to the MHC. They are experimental allergic encephalitis (Gasser et al., 1973; Williams and Moore, 1973), autoimmune complex nephritis (Stenglein et al., 1975) and autoimmune thyroiditis (Penhale et al., 1975; Rose, 1975).

It is interesting that autoimmune thyroiditis is associated with the MHC in mice (Vladitu and Rose, 1971) as well as chickens and rats.

n man there are two forms of autoimmune thyroiditis, Grave's disease nd Hashimoto's disease. Only Grave's disease has been associated with LA. Also, experimental allergic encephalitis has been claimed as an nimal model for human multiple sclerosis. Both diseases are associated ith the MHC.

Suggestive evidence that disease susceptibility is associated with the MHC has been claimed in two other species. In cattle, an association between MHC type and susceptibility to an ocular squamous cell carcinoma has been claimed (Caldwell and Cumberland, 1978). In dogs, transmissible venereal tumours were derived from two unrelated dogs. Both tumours typed identically with thirty-seven antisera directed against DLA specificities. Further, survival of transplanted tumours appeared to be associated with DLA type (Epstein and Bennett, 1973).

3. Transplantation

The major histocompatibility system of several species has been shown to have a significant effect on graft survival in several species. The very name major histocompatibility complex - derives from the effect of this system on graft survival.

In mice, any one of three genes in the H-2 complex can cause rapid skin graft rejection. They map in the H-2K, H-2D and I-A or I-B regions. At the moment it is not known if the H-2K and H-2D loci themselves influence graft survival or if the effect is due to closely linked genes. The role of H-2 system in graft survival has been reviewed by David (1977).

In man, matching for HLA-A and HLA-B antigens has been shown to have a definite influence on graft survival. This effect is more pronounced in intrafamily grafts than when grafts are exchanged between unrelated individuals. These findings have led to the hypothesis that the genes influencing transplantation are not HLA-A and HLA-B but genes in very close linkage (reviewed by Morris et al., 1978). Oliver et al. (1972) suggested that matching for HLA-B antigens was more important than matching for HLA-A antigens. This observation has subsequently been confirmed by the same group (Festenstein, 1978). This is consistent with the hypothesis that linked genes are responsible for graft rejection and implies that the relevant genes are more closely linked to HLA-B than to HLA-A. Solheim et al. (1977) have claimed that matching for HLA-C antigens is not important. This result is not consistent with the linked gene hypothesis. However, data from only a small number of graft recipients were analysed by Solheim et al. and the finding has still to be confirmed.

Graft survival, especially within families, has been shown to be influenced by the MHC in rhesus monkeys (Balner et al., 1971b). dogs (Vriesendorp et al., 1971, 1977), pigs (Vaiman et al., 1970b; Iványi, 1977a), chickens (Schiermann and Nordskog, 1971; Hála, 1977), guinea pigs (de Weck et al., 1971) and rabbits (Iványi, 1977b). In guinea pigs (de Weck et al., 1971), MHC compatibility was shown to cause detectable prolongation of graft survival amongst grafts exchanged between unrelated as well as related animals. This could be due to significant linkage disequilibrium between the genes detected serologically and the genes responsible for graft survival.

Suggestive evidence for a role of the MHC in graft survival has also been obtained in chimpanzees (Balner, 1977), hamsters (Duncan and Streilein, 1976) and Xenopus laevis (Du Pasquier et al., 1975).

Summary

The purpose of this review was two-fold. Firstly, to examine the work which has been carried out on the major histocompatibility complex in different species and secondly, to examine the areas of similarity and dissimilarity between species. There is extensive similarity between species but there are also dissimilarities. For example, the arrangement of different loci in the mouse H-2 system differs from the arrangement observed in men, rhesus monkeys, dogs and pigs. It appears reasonable to use the work carried out in other species as a model for the study of the sheep MHC. Although it seems sensible to be aware that differences may exist.

In this thesis, I set out to look at the genetic control of sheep lymphocyte antigens. The work performed in other species suggests that the majority of lymphocyte antigens will be controlled by a single genetic system. Although other loci may also exist. Within the system the antigens are expected to form mutually exclusive series. Each series is probably coded for alleles of a single locus. At least three loci are expected. It is also possible that more than one antigen may be present on the same allelic product.

CHAPTER 2

THE FREEZING OF SHEEP LYMPHOCYTES

Introduction

Materials and Methods

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2. Comparison of Fresh and One Day Old Blood from Different Breeds.
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4. The Effect of Slow Addition of DMSO on the Percentage Viability and Percentage Recovery.
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6. The Effect on Percentage Viability and Percentage Recovery of Sheep Lymphocytes when Varying the Thawing Parameters.
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9. Recovery of Lymphocytes from Whole Blood Before and After Freezing.
10. The Use of Cryopreserved Cells in Cytotoxicity Testing
    a) The Effect of Freezing and Thawing on the Sensitivity of Sheep Lymphocytes to Lysis in a Type II Micro-lymphocytotoxicity test.
    b) The Effect of Freezing and Thawing on Specific lymphocyte Antigens.

Discussion

Introduction

The use of cryopreserved lymphocytes for cytotoxicity testing is well established in other species, notably the human (Stopford et al., 1972; Wood et al., 1972). This technique offers many advantages. These advantages include comparison of cells and reagents over time, ease of handling, setting up of a greater number of tests if desired and finally convenience. In sheep, where regular access to animals is not always possible and where time of bleeding is often necessarily late in the day, and also where there is a large turnover of animals, especially rams, the importance of cryopreservation is even more apparent. Accordingly, it was decided to develop a cryopreservation technique for sheep lymphocytes.

A literature search was made with the aid of MEDLINE. This revealed a wide variety of techniques of which several were attempted, including those of Hors et al. (1973) and Farrant et al. (1974). The Farrant technique was chosen for further study. Its advantages included its ease, simplicity and the fact that no expensive equipment was necessary. Initial tests showed that cells could be preserved using this method. For cytotoxicity testing, cell populations must be of high purity and viability. If cryopreservation is to be of use, the recovered cell population must meet these standards of purity and viability and additionally give reasonable recoveries and results which must be reproducible.

In an attempt to improve the performance of sheep lymphocytes in this test, a number of the parameters were investigated.

The parameters investigated included the method of adding DMSO, the percentage of DMSO used in the freezing process, time at the holding temperature of -26°C. and the composition of the thawing medium. These changes allowed the development of a suitable protocol for the preservation of sheep lymphocytes for cytotoxicity testing.

The aim of this chapter is to describe the methods used, the results of investigating a number of the parameters, the modifications made to Farrant's procedures and the results obtained with the modified test.

Materials and Methods

Lymphocyte Separation

The technique used in cytotoxicity testing for the isolation of viable, very pure lymphocyte populations was also employed here. Slight modifications were made to allow a greater recovery of cells. Depending upon the availability of whole blood and the requirements of the test, 2-12 mls. of whole blood were used. No more than 3 mls. of blood was layered in any one tube. When 2-4 tubes were used, samples were pooled (two into one) after centrifugation on Ficoll-Hypaque and two into one again, if necessary, after the first washing step. All other steps were those described in Chapter 3. The cell concentration, purity and viability were assessed as before. All suspensions were prepared in 1 ml. HBSS + 10% NRSS (Hank's Balanced Salt/

Salt Solution, GIBCO Biocult, Scotland, no sodium bicarbonate was added. A pool of non-reactive ram's serum at a final concentration of 10% was used as a buffer.). No adjustments were made to the final volume of 1 ml.

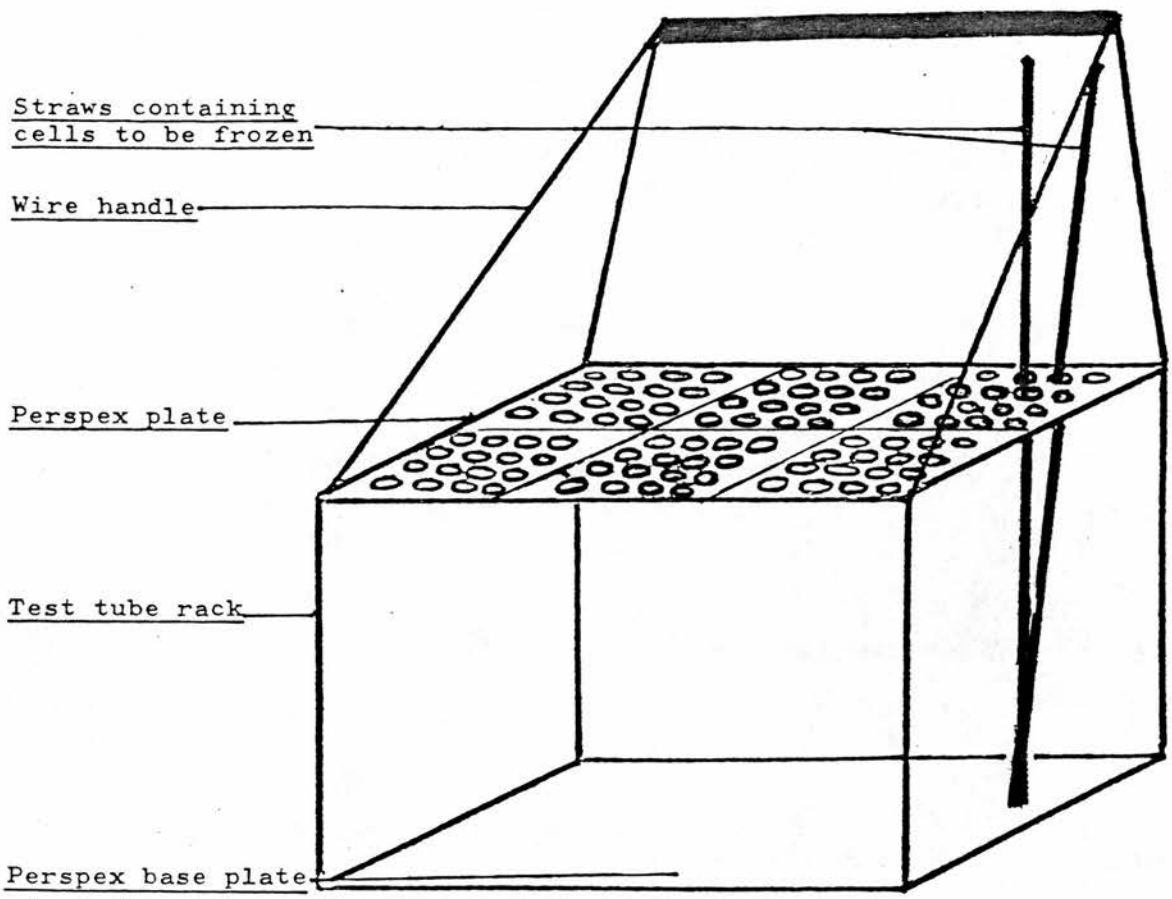
Freezing

The cell suspension and the DMSO (dimethylsulphoxide, BDH, England) solution in phosphate buffered saline(PBS) pH 7.2 were transferred to a cold room. This cold room was kept at a temperature of 2-6°C. Both suspensions were allowed 10 minutes to equilibrate. All subsequent steps were carried out in this cold room.

The subsequent procedures described below were those followed initially. Modifications to the procedure were made and these are described later. An equal volume of the 10% DMSO solution was added to the cell suspension. After mixing with a vortex mixer, the cell suspension in 5% DMSO was rapidly sucked up into 8 x 0.25 ml. plastic straws (IMV L'Aigle, France). The straws were then sealed in polyvinyl acetate (IMV L'Aigle, France) and subsequently placed into a freezing basket made to our own specifications (Fig. 1). The basket was then plunged into a bath of industrial methylated spirit. The bath was kept at -26°C. at the bottom of a commercial deep freeze. After 10 minutes, the straws were removed, partially dried with tissue and plunged into a liquid nitrogen bath (-196°C.), prior to permanent filing in liquid nitrogen.

Figure 1

Diagram of the Freezing Basket



Thawing

All procedures were carried out at room temperature (10-24°C.) except those involving the 37°C. water bath.

Prior to thawing, test-tubes containing 4 mls. of the thawing medium and the stock bottle of HBSS + 10% NRSS were warmed in the 37°C. water bath.

The cell suspension was rapidly thawed by removing from liquid nitrogen and agitating for 5-8 seconds in the water bath. The straws were then cut at both ends and the contents run into the thawing medium. The straws were flushed through and the test-tubes filled with HBSS + 10% NRSS. The tubes were then spun at 100 g. for 15 minutes to pellet the lymphocytes. The supernatant was then discarded and the cells resuspended in either 0.5 mls. or 1.0 mls. HBSS + 10% NRSS.

Initially, the thawing medium was 2 mls. NRSS + 2 mls. HBSS + 10% NRSS.

Assessment of Recovery and Viability

The purpose of the cell freezing was to preserve lymphocytes for cytotoxicity testing. The main requirement of cytotoxicity testing is a suspension of high viability and purity. Viability, purity and percentage recovery were assessed by phase contract microscopy with Neubauer haemocytometers. (Throughout this chapter, the percentage recovery refers to the recovery of viable cells expressed as a percentage of the input of viable cells.) This method has disadvantages. It is possible that although cells have the appearance of/

of living cells, they may not function normally (e.g. respond to mitogens (Knight and Farrant, 1978)). However, as our assay uses lymphocytes solely as targets for antibody, this point was not felt to be relevant to this study.

The behaviour of cryopreserved cells in microlymphocytotoxicity tests is described later in this chapter. When using thawed cell suspensions, the cell concentration was adjusted as necessary.

After testing a number of parameters, changes were made in the freezing and thawing procedures. Before describing the experiments it may be useful to outline the protocol which was adopted for routine use.

Routine Freezing and Thawing Procedures

The collection of blood by jugular venepuncture into dry tubes coated with Lithium Heparin is described in Chapter 3.

To give sufficiently high lymphocyte recoveries, it was necessary to use 12 mls. of whole blood for each animal. Four separate 3 ml. lots of heparinised blood were each layered onto 2.5 mls. of Ficoll-Hypaque (specific gravity 1.064). No more than 16 different preparations were made at any one time.

Cells were prepared as described in Chapter 3. The four white-cell rich interfaces obtained after centrifugation on Ficoll-Hypaque were pooled into two MSE 110 x 16 mm. conical-bottomed centrifuge tubes.

After the first wash, cells were resuspended in 2 mls. HBSS + 10% NRSS. Lymphocyte concentration, purity and viability were assessed by phase contrast microscopy. Experiments to determine lymphocyte recovery with this technique are described below.

Cell suspensions and a 34% DMSO suspension in PBS (pH 7.2) were then transferred to a cold room at 4°C. Both suspensions were allowed 10 minutes to equilibrate. 1 ml. of the DMSO solution was slowly added to the 1 ml. cell suspension. The cell suspension was thoroughly mixed on a mechanical mixer during this addition. The suspension was then sucked into straws. The straws were sealed with polyvinylacetate and rapidly transferred to a freezing basket.

This freezing basket was immersed in a bath of industrial methylated spirits at -26°C. After 10 minutes, the straws were removed, partially dried with tissue and rapidly transferred to pre-chilled polygons and then into liquid nitrogen.

Cells were stored in liquid nitrogen until thawing.

When required, cells were thawed. One test-tube was used for each animal. Empty Becton-Dickinson 100 x 13 mm. round-bottomed vacutainer tubes were used. Test tubes containing 4 ml. NRSS (a pool of heat inactivated non-reactive ram's serum) and a stock bottle of HBSS + 10% NRSS were warmed in a 37°C. water bath. Straws were removed from liquid nitrogen and rapidly thawed by agitating for 5-8 seconds in the water bath. Straws were cut at both ends and the contents run into a test-tube. Straws were then flushed through with HBSS + 10% NRSS.

The thawed cell suspension was washed by centrifugation at 100 g. for 15 minutes. The supernatent was discarded and the cell pellet resuspended in 0.5 mls. HBSS + 10% NRSS. Concentration, purity and viability were assessed as before by phase contrast microscopy. If the cells were to be used for cytotoxicity testing and if purity and viability were acceptable, lymphocyte concentrations were then adjusted to 1.5×10^6 lymphocytes/ml. (In practice viability and purity seldom fell below permitted minima.)

Results

1. The Effect of Different Holding Times at -26°C. Before Freezing in Liquid Nitrogen

The experiment was set up to look at the effect of holding cells for different lengths of time at -26°C. before freezing them in liquid nitrogen. Four Welsh mountain sheep were bled in the usual manner. Lymphocytes from 8 mls. of blood from each animal were separated, pooled and counted. Eight straws were prepared for each animal. Two straws from each animal were kept at -26°C. at each of four different times; 2 minutes, 10 minutes, 30 minutes and 60 minutes. The results are presented below in Table 1.

In these results, the percentage viability was assessed but not recorded. Different preparations showed differences in viability. Preparations made after only 2 minutes at -26°C. had a higher background cytotoxicity than other preparations. Due to this variation in viability, all future preparations were scored both for viability and recovery.

Table 1

The Effect of Different Holding Times at -26°C. on the Freezing of Sheep Lymphocytes

Sheep Identity	Time at -26°C. (Minutes)	Viable Lymphocytes				Percentage Recovery
		Before Freezing		After Freezing		
		Cell Concentration x 10^{-4}/ml.	Percentage Viability	Cell Concentration x 10^{-4}/ml.	Percentage Viability	
3R559	2	295	96	25	N.A.	34
	10			28	N.A.	38
	30			23	N.A.	31
	60			22	N.A.	30
3R531	2	690	100	63	N.A.	37
	10			109	N.A.	63
	30			68	N.A.	39
	60			68	N.A.	39
8R475	2	570	100	63	N.A.	44
	10			56	N.A.	39
	30			55	N.A.	39
	60			43	N.A.	30
3R454	2	430	100	41	N.A.	38
	10			32	N.A.	30
	30			34	N.A.	32
	60			16	N.A.	15
Summary	2					38
	10					43
	30					35
	60					26

Key:- N.A. = Not Assessed

These experiments show, for holding times between two and sixty minutes, that the length of time at -26°C. has very little effect on cell recovery. A ten minute holding time appears slightly better but this may be due to chance effects. As ten minutes is a convenient time, and as this is recommended by Farrant et al. (1974) in the paper describing the technique, this time was used for all future tests.

2. Comparison of Fresh and One Day Old Blood from Different Breeds

Lymphocytes from twenty-four sheep were frozen and thawed. These sheep were eight unrelated sheep from each of two breeds and a cross-breed (Cheviot, Border Leicester and Finnish Landrace x Dorset Horn). Samples from the same breed were frozen fresh (separation procedures started two hours after sampling) and also at a day old (separation procedures started 22 hours after sampling). Two to three mls. of blood were used for each preparation.

The purpose of this was three-fold: a) to set a baseline to compare subsequent tests; b) to see if any gross differences existed between different breeds; and c) to look at the effect of ageing on our samples.

The results are set out in Table 2.

The N.A. results for 4FD15 is due to the recorded recovery being slightly greater than 100%. The two other N.A. results are due to the experiments not being carried out. Results from these two are not included in the subsequent comments. These results suggest that there are no great differences in freezing and thawing with fresh and day old blood/

Table 2

Comparison of Fresh and One Day Old Blood from Different Breeds

Breed	Sheep Identity	FRESH BLOOD				24 hour OLD BLOOD			
		Lymphocytes/ml. x 10^{-4}				Lymphocytes/ml. x 10^{-4}			
		Fresh	Thawed	Percentage Viability	Percentage Recovery	Fresh	Thawed	Percentage Viability	Percentage Recovery
Cheviot	4C27	230	86	82	37	135	53	84	39
	83C02	195	34	69	17	275	89	90	32
	94C60	125	9	82	7	75	39	80	52
	94C61	255	17	65	7	245	106	94	43
	95C00	215	41	82	19	575	70	93	12
	5P112	275	30	77	11	150	40	82	27
	5P349	240	25	53	10	235	66	62	28
	95C60	265	49	61	18	255	86	84	34
Finn x Dorset	582	165	120	96	73	35	9	90	26
	925	125	58	91	46	195	37	90	19
	095	175	58	89	33	60	9	82	15
	286	165	17	71	10	70	17	85	24
	3G77	20	12	92	60	125	32	94	26
	4G105	100	29	94	29	65	33	97	51
	4FD3	30	16	100	53	45	5	63	11
	4FD15	50	75	86	N.A.	45	12	86	27
Border Leicester	5L17	330	185	92	56	355	72	72	20
	5L22	230	91	88	40	250	93	88	37
	3L1	190	117	90	62	285	48	94	17
	2L1	280	44	66	16	425	109	85	26
	BL8	225	80	88	36	390	85	81	22
	BL9	175	147	91	84	150	30	86	20
	BL11	40	6	60	15	N.A.	N.A.	N.A.	N.A.
	6BL2	N.A.	N.A.	N.A.	N.A.	395	141	88	36

blood (mean viabilities 81% and 84%, mean recoveries 34% and 28%). Small differences may exist but they would not be detectable here. A similar conclusion can be drawn in respect of breed differences. (The mean percentage viabilities and percentage recoveries for the Cheviot, Finn x Dorset and Border Leicester were 72.5%, 88% and 83.5% and for recoveries 24.6%, 34.2% and 34.8% respectively. In view of the large variations between samples and the small size of the test, these differences do not appear to be significant.

There appears to be no great differences between breeds. The differences appear to be between individual animals. Thus, the experiments designed to investigate the parameters of the test, used animals from whatever breeds were conveniently available.

3. Handling Experiments

When using the Farrant technique to freeze cells, there are two possible causes for the loss of cells and a reduced recovery. These are: i) the freezing process itself, and ii) handling procedures. Loss of cells in the handling procedures may be brought about by not taking up the complete cell suspension into the plastic straws or by spillage when cutting the straws after thawing. A simulated freezing experiment was carried out to determine the percentage of lymphocytes lost due to the handling technique. Once this value was established, it was possible to assess the loss due to the freezing technique.

Additionally, the arrangement of straws was varied in an attempt to improve recoveries.

Methods

Cell separation, cell freezing and thawing were carried out as before.

Simulated Freezing

The prepared cell suspension was allowed 10 minutes at 4°C. to equilibrate. 1 ml. of 10% DMSO solution was added to 1 ml. of the cell suspension. This was thoroughly mixed and sucked up into eight plastic straws. The straws were set into a freezing basket and moved to a second surface. This mimicked the same handling as plunging the straws into a bath of industrial methylated spirits. The straws were left 10 minutes. After this they were removed from the basket, wiped and placed in polygons. The thawing process was then copied in its entirety.

Freezing Method with Variation of Straw Arrangement

Prepared cell suspensions were allowed 10 minutes at 4°C. to equilibrate. 1 ml. of 10% DMSO was added to 1 ml. of the cell suspension and mixed with a vortex mixer.

The six cell suspensions in DMSO were now treated in one of three ways.

i) Part of the suspension was sucked up with six straws and the remainder was sucked up, as far as possible, with two more straws. This was repeated for a second suspension.

ii) A third suspension was partly sucked up using seven straws and the remainder was then sucked up with one straw. This was repeated with a fourth suspension.

iii) A fifth suspension was partly sucked up using seven straws and the remainder was sucked up with two straws. This was also repeated with a sixth suspension.

Straws were then sealed and frozen as outlined above.

Results

The results (Table 3) show that the handling procedures have little effect on cell viability. These results suggest that DMSO is not toxic for sheep lymphocytes in this system, i.e. at a 5% final concentration in 5% serum at 4°C. This is in agreement with other (unreported) observations made by me in the course of freezing studies. DMSO, even at a final concentration of up to 20%, is not toxic for sheep lymphocytes in serum, even when left up to thirty minutes at room temperature.

Secondly, these results show that there is a significant loss of cells caused by handling procedures. In this experiment, 25.7% of cells were lost on average. Even more important, the proportion of cells recovered varied widely from only 36% in cell 4S8 to 99% in cell 5S23 (Table 3).

In an attempt to minimise this loss, straws were in future cut very near the ends to remove a minimal amount of cells. Also, the arrangement of straws was varied. Previously, in accordance with the procedure in the cytotoxicity test, 100 x 13 mm. round-bottomed test-tubes were used for the washing steps. Subsequently, MSE 110 x 16 mm. conical test-tubes were used. This made any remaining cell suspension, after sucking-up, more noticeable. Also, any remaining suspension could be extracted more easily.

The straw arrangement has been explained earlier. The arrangement of 7 + 1 gives the highest percentage recovery (Table 4). The final drops of the cell/

Table 3

Simulated Freezing of Sheep Lymphocytes

Sheep Identity	Blood (mls.)	Lymphocytes per ml. ($\times 10^{-4}$)				Percentage Recovery
		Before simulated freezing		After simulated freezing		
		Cell concentration $\times 10^{-4}$/ml.	Percentage viability	Cell concentration $\times 10^{-4}$/ml.	Percentage viability	
4S2	6	595	100	405	100	68
4S8	6	535	99	190	100	36
5S7	6	615	98	530	99	86
5S15	6	445	100	360	100	81
5S22	6	575	100	365	100	63
5S23	6	385	100	380	100	99

Table 4

The Arrangement of Straws

Sheep Identity	Straw Arrangement	Live Lymphocytes				Percentage Recovery
		Before Freezing		After Freezing		
		Cell concentration x 10^{-4}/ml.	Percentage viability	Cell concentration x10^{-4}/ml.	Percentage viability	
4S2	6 + 2	560	100	104	87	19
4S8	7 + 1	615	100	312	79	51
5S7	7 + 2	550	100	171	76	39
5S15	7 + 2	610	100	264	82	42
5S22	6 + 2	640	100	333	80	52
5S23	7 + 1	230	100	248	73	N.A.

cell suspension are more easily picked up using a single straw than by using two straws. This result reflects this observation. 8 straws did not pick up the complete cell suspension, this would cause a low recovery. 9 straws in an 8 + 1 arrangement were used in future tests.

These modifications minimised all conceivable losses due to handling. Attempts to improve the percentage recovery and the percentage viability were subsequently made by improving the conditions for the freezing process.

4. The Effect of Slow Addition of DMSO on the Percentage Viability and Percentage Viability and Percentage Recovery

When DMSO is added to a cell suspension, energy is released in the form of heat. This could conceivably be a cause of cell death. If the heat change could be made less drastic, the effect could be minimised. The purpose of this experiment was to see if the percentage viability of the thawed suspension and the percentage recovery could be improved by the slow addition of DMSO.

Two experiments to test this were carried out. In the first experiment, four preparations were made from each of four sheep. In two preparations 10% DMSO was added slowly, while mixing thoroughly to dissipate heat. In the other two preparations, 10% DMSO was added before mixing in the usual manner, (i.e. all at once). All other procedures were as outlined above. Results are set out below, in Table 5.

Table 5

Slow Addition of DMSO (1)

Sheep Identity	Slow Addition of DMSO	Viable Lymphocytes				Percentage Recovery
		Before Freezing		After Thawing		
		Cell concentration x 10^{-4}/ml.	Percentage viability	Cell concentration x 10^{-4}/ml.	Percentage viability	
582	No	170	100	119	44	11
582	No	195	100	17	52	9
582	Yes	185	100	20	54	11
582	Yes	185	100	38	83	21
286	No	80	100	3	23	3
286	No	235	100	18	42	8
286	Yes	80	100	14	74	18
286	Yes	275	100	62	86	23
4FD3	No	110	100	9	50	8
4FD3	No	60	100	21	81	35
4FD3	Yes	105	100	23	68	22
4FD3	Yes	65	100	32	74	49
4FD15	No	110	100	6	24	5
4FD15	No	145	100	15	41	10
4FD15	Yes	115	100	34	72	30
4FD15	Yes	145	100	37	57	26

From this table, the following were calculated:-

(i) Mean percentage viability for slow addition of DMSO = 71%

(ii) Mean percentage recovery for slow addition of DMSO = 25%

(iii) Mean percentage viability for normal addition of DMSO = 45%

(iv) Mean percentage recovery for normal addition of DMSO = 12%

From the above results, slow addition of DMSO before freezing shows an improvement on both the percentage viability and the percentage recovery. However, in past freezing experiments, the percentage viability for normal addition of DMSO has ranged between 70-95%. An unusual amount of cells were dead on recovery after freezing. This may have been due to lack of correct storage. Due to the balanced design of the experiment, the low viabilities recorded do not invalidate the conclusion that slow addition of DMSO is beneficial to cell viability and cell recovery. However, this experiment was repeated. This second experiment is now described.

Two lymphocyte preparations were made for each of four animals. One preparation of 10% DMSO was added slowly while mixing thoroughly. In the other preparation 10% DMSO was added before mixing. All other procedures were those outlined above. This test was essentially a repetition of the above with no replicates. These results are presented in Table 6.

The results show a slight superiority for the slow addition of DMSO. For the slow addition and the normal addition, the mean percentage viabilities are 81% and 77% respectively. Similarly, the mean percentage recoveries are 40% and 27%.

Table 6

Slow Addition of DMSO (2)

Sheep Identity	Slow Addition of DMSO	Viable Lymphocytes				Percentage Recovery
		Before Freezing		After Freezing		
		Cell concentration x 10^{-4}/ml.	Percentage viability	Cell concentration x 10^{-4}/ml.	Percentage viability	
BL8	No	250	100	85	85	34
BL8	Yes	210	100	73	86	35
6BL19	No	215	98	35	74	16
6BL19	Yes	200	100	51	86	26
5L25	No	180	100	68	80	38
5L25	Yes	220	100	62	66	28
5L27	No	140	98	30	67	21
5L27	Yes	110	96	77	84	70

Both experiments have shown a slight superiority for the slow addition of DMSO. Therefore, it appears likely that slow addition is beneficial in cell freezing. Certainly, slow addition is unlikely to be harmful. Also, the slow addition of DMSO is recommended by several authors (e.g. Amos, 1976; Fuller, 1976). On the basis of this and the two previous experiments, DMSO was always added slowly, while mixing, in subsequent experiments.

5. The Effect of Altering the Lymphocyte Concentration on the Percentage Recovery and the Percentage Viability

The aim of the following two experiments was to see if the concentration of lymphocytes frozen affected the recovery or viability of the thawed population.

Very high concentrations of cells were not used. This was for two reasons. Firstly, taking large volumes of blood from sheep is not feasible as a routine technique. Whilst large volumes could be taken for this test, it did not seem sensible to develop a technique which could not be used. Nor did it appear sensible to look at parameter values which would not be achieved in normal use. Secondly, it is difficult to prepare cells from large volumes without incurring high granulocyte contamination in some samples. Several authors have indicated that a high proportion of granulocytes adversely affects freezing, (e.g. Fuller, 1976). Therefore high concentrations might be expected to reflect both the effect of high concentration and granulocyte contamination.

Eight 3 ml. preparations were made from each of three animals.

Lymphocytes/

Lymphocytes were prepared in the routine way to give four preparations. Cell concentrations for each animal were approximately adjusted to the following values:-

i) 7×10^6 lymphocytes/ml.

ii) 4×10^6 lymphocytes/ml.

iii) 1×10^6 lymphocytes/ml.

iv) 0.5×10^6 lymphocytes/ml.

The four preparations from each animal were frozen and thawed in the routine manner. Results are tabulated below (Table 7).

The results from Table 7 are plotted on a graph in Figures 2 and 3.

These results show that there is a decrease in the percentage recovery of thawed cells with an increase in lymphocyte concentration. The percentage viability is not greatly affected by cell concentration. The differences are not large and these experiments could be due to chance effects. Consequently, this experiment was repeated.

In the second experiment, 3 or 4 preparations were made from four animals. Cell concentrations were adjusted to differ widely. These results are summarised in Table 8. Viability and recovery are plotted against lymphocyte concentration in Figures 4 and 5.

This second experiment shows that percentage recovery decreases as cell concentration increases. Cell viability is not affected. This confirms the results obtained in the first experiment.

This result presented something of a dilemma. As cell concentration increased, the total recovery increased but the percentage recovery fell. It did not seem sensible to adjust cell concentrations to improve recovery. In subsequent experiments, cell concentrations were not adjusted.

Table 7

The Effect of Cell Concentration on Recovery and Viability (1)

Sheep Identity	Lymphocyte Concentration x 10^{-6}/ml.	Viable Lymphocytes				Percentage Recovery
		Before Freezing		After Thawing		
		Cell concentration x 10^{-6}/ml.	Percentage viability	Cell concentration x 10^{-6}/ml.	Percentage viability	
4S2	6.45	646	100	129	85	20
4S2	3.95	395	100	116	82	29
4S2	1.30	130	100	24	71	18
4S2	0.80	80	100	26	76	33
5S15	7.30	730	100	149	81	20
5S15	3.30	330	100	96	88	29
5S15	0.55	55	100	24	83	44
5S15	0.45	45	100	18	90	40
5S22	5.75	575	100	289	88	50
5S22	4.80	480	100	193	85	40
5S22	0.65	65	100	44	94	68
5S22	0.30	35	100	24	92	80

Figure 2

Graph showing the percentage viability against concentration

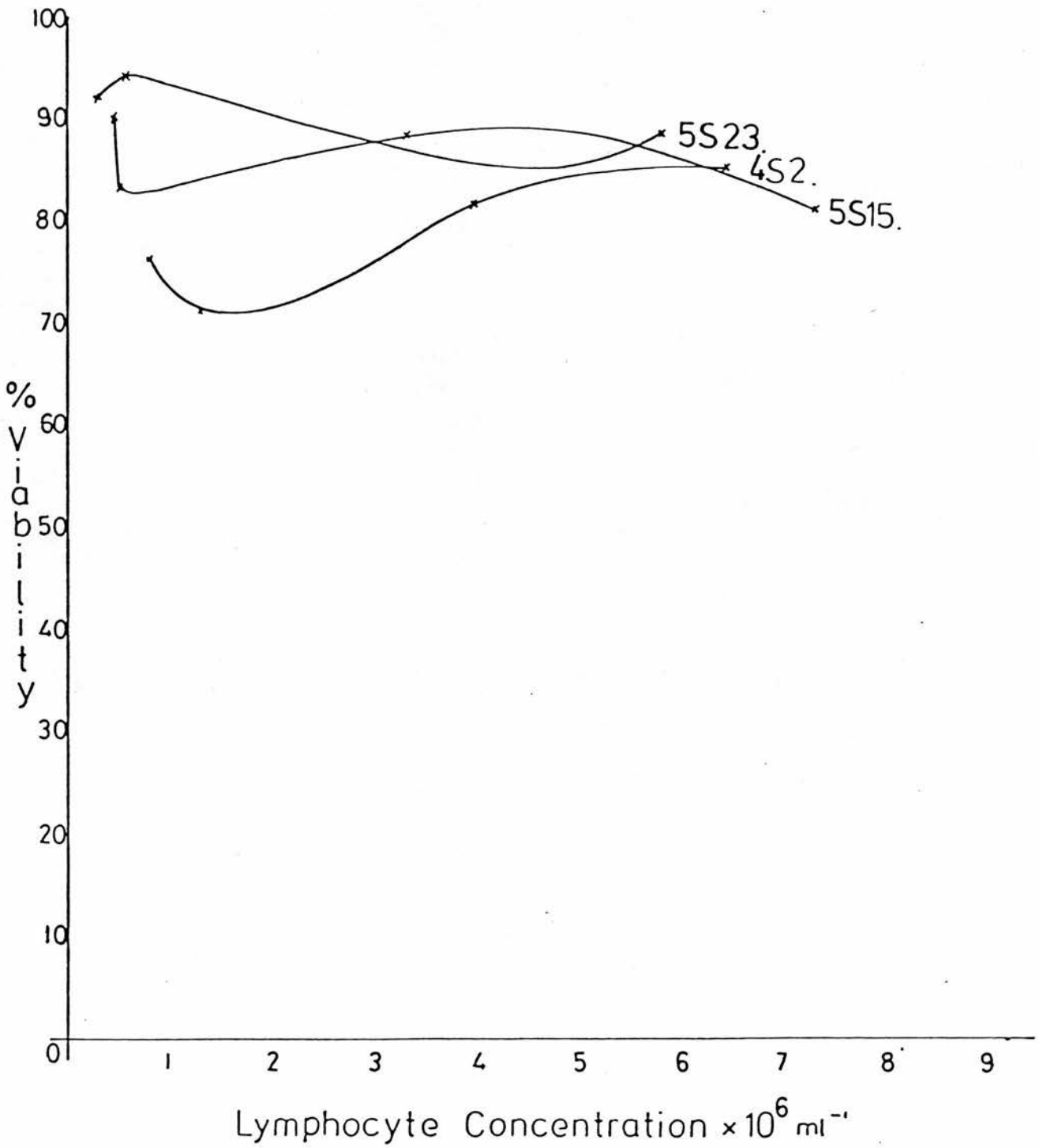


Figure 3

Graph showing the percentage recovery against concentration

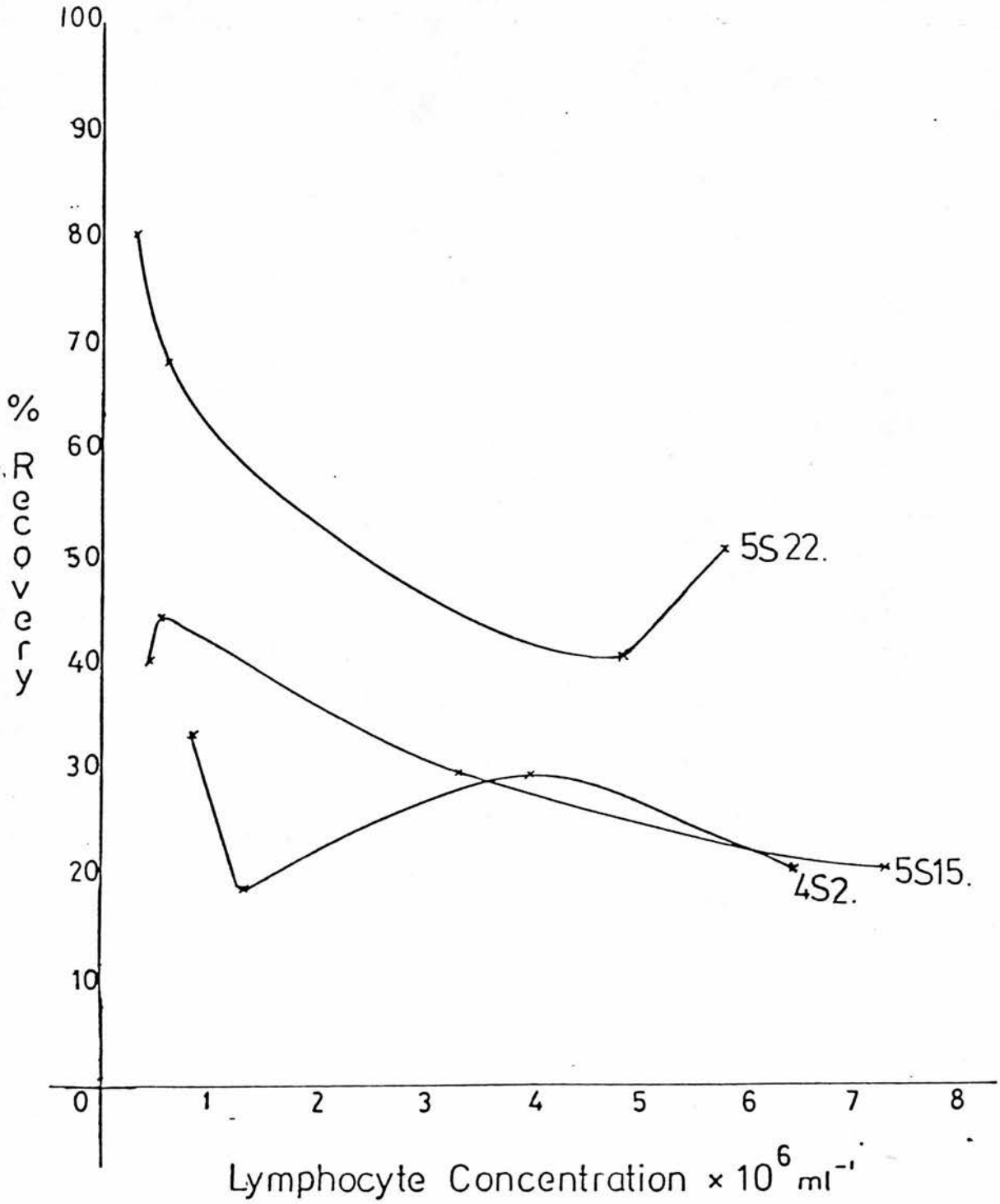


Table 8

The Effect of Cell Concentration on Recovery and Viability (2)

Sheep Identity	Lymphocyte Concentration x 10^{-6}/ml.	Before Freezing		After Thawing		Percentage Recovery
		Lymphocyte Concentration x 10^{-6}/ml.	Percentage viability	Lymphocyte Concentration x 10^{-6}/ml.	Percentage viability	
4S2	4.45	445	100	96	82	22
4S2	2.20	220	100	75	85	34
4S2	0.65	65	100	36	90	55
5S15	4.7	470	100	190	94	40
5S15	2.3	230	100	136	91	59
5S15	1.35	135	100	57	84	42
5S22	2.55	255	100	70	84	27
5S22	2.3	230	100	121	90	53
5S22	1.8	180	100	103	90	57
5S23	6.9	690	100	362	96	52
5S23	3.0	300	100	300	99	47
5S23	1.55	155	100	155	95	65
5S23	0.95	95	100	95	98	41

Figure 4

Graph showing the percentage viability after thawing against original cell concentration

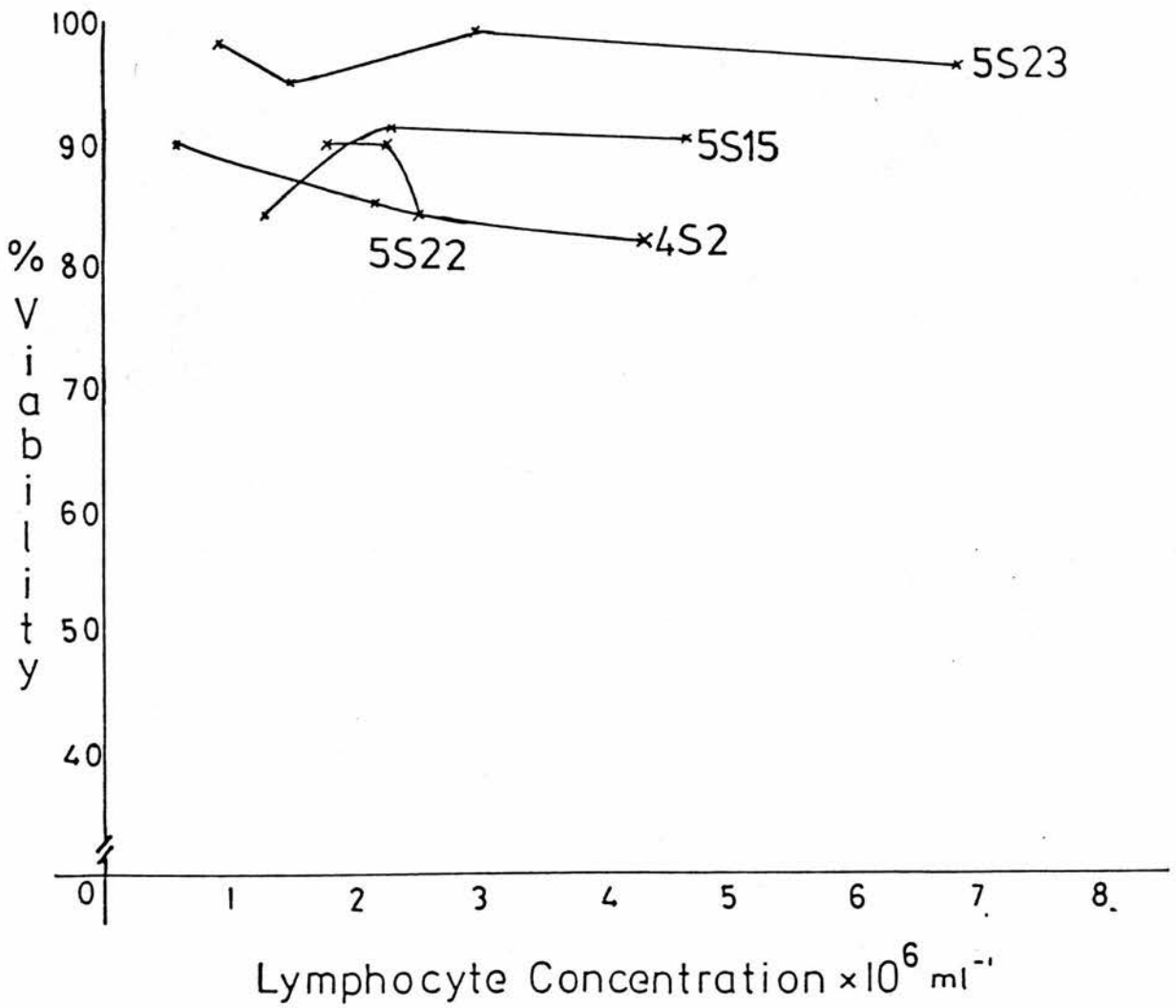
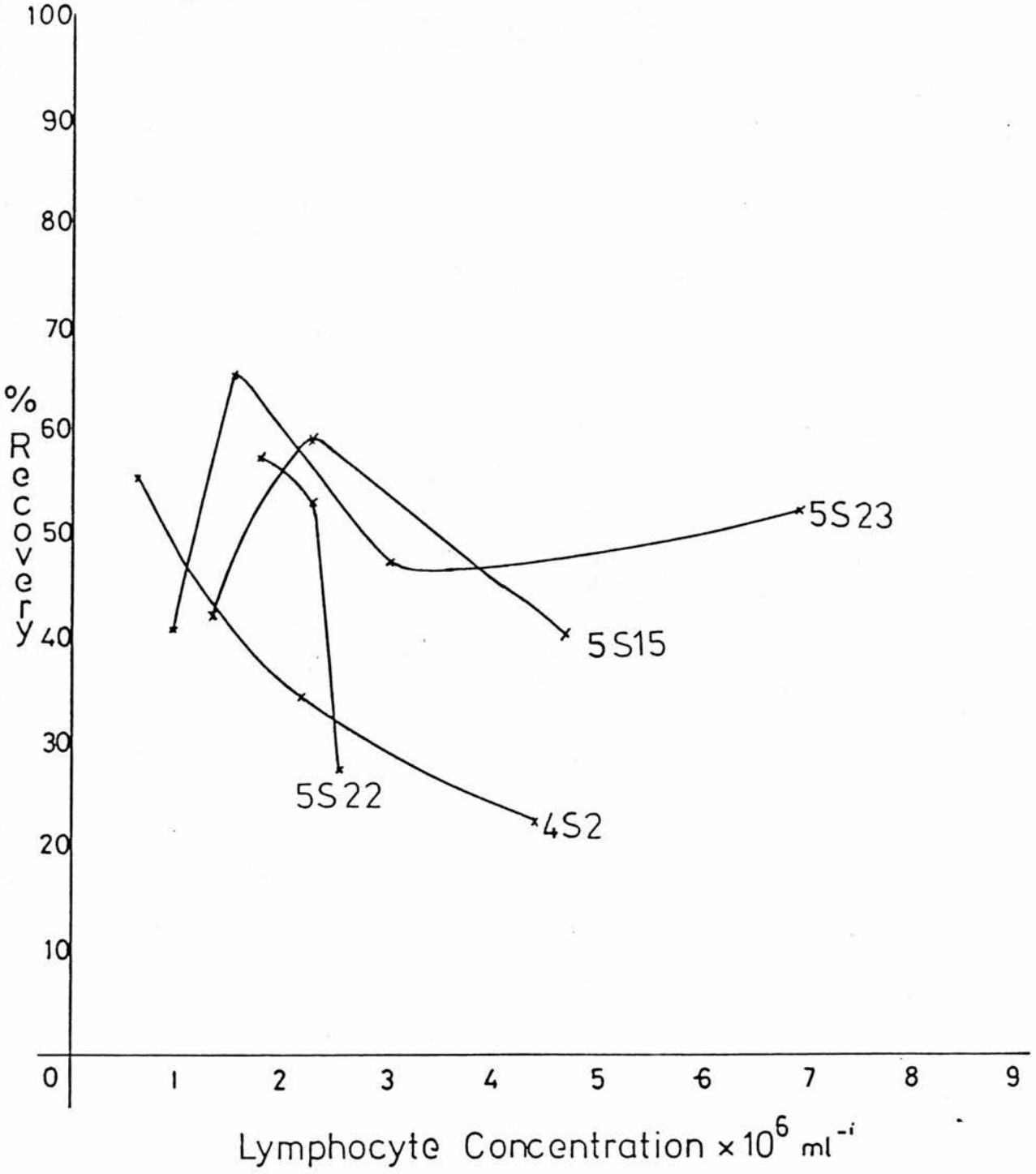


Figure 5

Graph showing the percentage recovery after thawing against original cell concentration



6. The Effect on Percentage Viability and Percentage Recovery of Sheep Lymphocytes When Varying the Thawing Parameters

Two experiments were carried out. The first was to determine the optimum mixture of HBSS + 10% NRSS with non-reactive ram's serum (NRSS) in the thawing. In the second experiment, the thawed cells were left for 30 minutes before washing to see if this could improve the viability and recovery of the thawed cell population.

For the first experiment, lymphocytes from four animals were prepared and frozen as described above. A total of 36 straws per animal were frozen. On thawing, the frozen cells were divided into four equal sets of 9 straws. Each set was thawed and suspended in a different thawing medium. These are described in Table 9.

The percentage viability and recovery were calculated in the usual way. The mean value was also calculated for each medium.

From the above results, and taking the mean values, it can be seen that thawing in 4 mls. of non-reactive serum, gives the greatest percentage viability. The percentage recovery is not the greatest value but this is not as important as viability for cytotoxicity testing. Hence, it was decided to use 4 mls. of serum in all subsequent experiments.

In the second experiment, cells left 30 minutes in 4 mls. serum + 2 mls. HBSS + 10% NRSS before washing, showed no significant differences in viability or recovery. Consequently, the prior procedure of washing immediately after thawing was followed in all subsequent experiments. Results are presented in Table 10. Unfortunately, it was not possible to use cells from the same animal in both treatments as insufficient volumes of blood were available.

Table 9

The Viability and Recovery of Frozen Cells

When Thawed in Different Thawing Media

Sheep Identity	Thawed in 4 mls. HBSS + 10% NRSS		Thawed in 3 mls. HBSS + 10% NRSS + 1 ml. NRSS		Thawed in 3 mls. NRSS + 1 ml. HBSS + 10% NRSS		Thawed in 4 mls. Non-Reactive Serum	
	Percentage viability	Percentage recovery	Percentage viability	Percentage recovery	Percentage viability	Percentage recovery	Percentage viability	Percentage recovery
6C7	87	49	83	42	92	81	98	63
6C22	89	56	93	51	89	46	95	30
7DF11	93	58	95	52	91	51	96	82
7DF12	93	76	87	60	86	100	97	44
Mean Percentage	90.5	59.8	89.5	51.3	89.5	69.5	96.5	54.8

Table 10

The Effect of Leaving Cells in Suspension for 30 Minutes before Washing

Sheep Identity	TREATMENTS			
	Normal		Left for 30 minutes	
	Percentage viability	Percentage recovery	Percentage viability	Percentage recovery
Border Leicester	90	72	-	-
Border Leicester	89	69	-	-
Border Leicester	81	77	-	-
Border Leicester	82	41	-	-
3R2	-	-	83	53
3R2	-	-	94	79
2A64	-	-	92	72
2A64	-	-	81	29
Mean Percentage	85.5	64.8	87.5	58.3

7. The Effect of Mixing Blood from Different Animals on the Percentage Viability and Percentage Recovery

To test a number of variables in the freezing technique, it was not always possible to obtain sufficient cells from different animals. Hence, pooled cell preparations were studied. Before this could be done, it was necessary to find out if pooling blood from different donors affected the percentage viability and recovery, as we would be using the improved freezing method on blood from single animals.

Two experiments were carried out. In each experiment 8 animals were used. Two single cell suspensions were prepared from each donor. Additionally, whole blood from these 8 animals was pooled and used to prepare 16 pooled cell suspensions. One cell suspension from each of the eight sheep and eight pooled cell suspensions were frozen at a final concentration of 15% DMSO. The remaining cell suspensions were frozen at a final concentration of 20% DMSO. All remaining treatments were those outlined above. Results are presented in Tables 11 and 12.

Tables 11 and 12 show that there is little difference between results obtained from pooled blood and results obtained with single donor samples. There is also some support for the assumption that mixed samples show less variation from the mean values.

These results suggest that there are no differences in behaviour on freezing with pooled and single donor suspensions. Consequently, pooled blood was used in future experiments when adequate supplies of individual samples were not available.

Table 11

Comparison of Pooled and Single Donor Lymphocytes Suspensions at Two DMSO Concentrations

Source of Lymphocytes	Final Percentage DMSO Concentration	Percentage Viability	Percentage Recovery
5L22	15	94	100
5L25	15	98	89
5L27	15	97	63
6BL19	15	94	86
6C13	15	97	48
6C19	15	98	37
6C22	15	99	64
7DF11	15	86	41
Mixture of above	15	98	83
"	15	93	53
"	15	99	80
"	15	86	70
"	15	98	75
"	15	97	62
"	15	97	72
"	15	98	95
5L22	20	97	95
5L25	20	97	90
5L27	20	98	73
6BL19	20	93	83
6C13	20	97	29
6C19	20	98	56
6C22	20	93	45
7DF11	20	97	39
Mixture of above	20	96	62
"	20	94	70
"	20	96	69
"	20	91	61
"	20	98	70
"	20	93	58
"	20	97	47
"	20	94	50

Table 12

Summary of Table 11

	Mean Percentage Viability	Mean Percentage Recovery
15% DMSO + Single Suspensions	95.4	66
15% DMSO + Mixed Suspensions	95.7	74
20% DMSO + Single Suspensions	96.25	65
20% DMSO + Mixed Suspensions	94.9	61

8. The Effect of Altering the DMSO Concentration on the Percentage Recovery and Percentage Viability

The aim of this series of experiments was to see how the percentage recovery and percentage viability of sheep lymphocytes were altered when the final DMSO concentration was varied.

Three experiments were carried out. In all three experiments pooled blood was used. The use of pooled blood allowed a greater number of replicates than would be possible using blood from a single donor.

Four replicate suspensions were tested at each DMSO concentration. In Experiment 1, final DMSO concentrations of 0-20% were used. In Experiments 2 and 3, cell behaviour was looked at in a narrower range of 14-25% final DMSO concentration. These results are summarised in Table 13 and Figures 6, 7, 8 and 9.

The results show that the percentage viability of the thawed lymphocytes population reaches a maximum when DMSO is used at a final concentration in the thawing medium of 16-17.5% The maximum percentage recovery lies between 16-18% final DMSO concentration.

In all subsequent freezing, a final DMSO concentration of 17% was used.

Cells prepared by the methods outlined above routinely gave a recovery of >40% with a viability >95%. There was no detectable background lysis in over 95% of samples tested. The other samples gave a high background lysis of ~5%. These viabilities are acceptable for microlymphocytotoxicity tests.

Table 13

DMSO Concentration and Cell Viability and Recovery

in Thawed Cell Suspensions

Final DMSO Concentration	Percentage Viability			Percentage Recovery		
	Experiment I	Experiment II	Experiment III	Experiment I	Experiment II	Experiment III
0.0	14	-	-	2	-	-
5.0	75	-	-	75	-	-
10.0	86	-	-	91	-	-
14.0	-	92	-	-	66	-
15.0	94	-	-	91	-	-
16.0	-	95	-	-	84	-
17.5	-	-	95	-	-	75
18.0	-	91	-	-	84	-
20.0	94	92	93	66	62	77
22.5	-	-	93	-	-	50
25.0	-	-	84	-	-	50

Key: - Not Tested

Figure 6

Graph showing the percentage viability against final percentage DMSO concentration

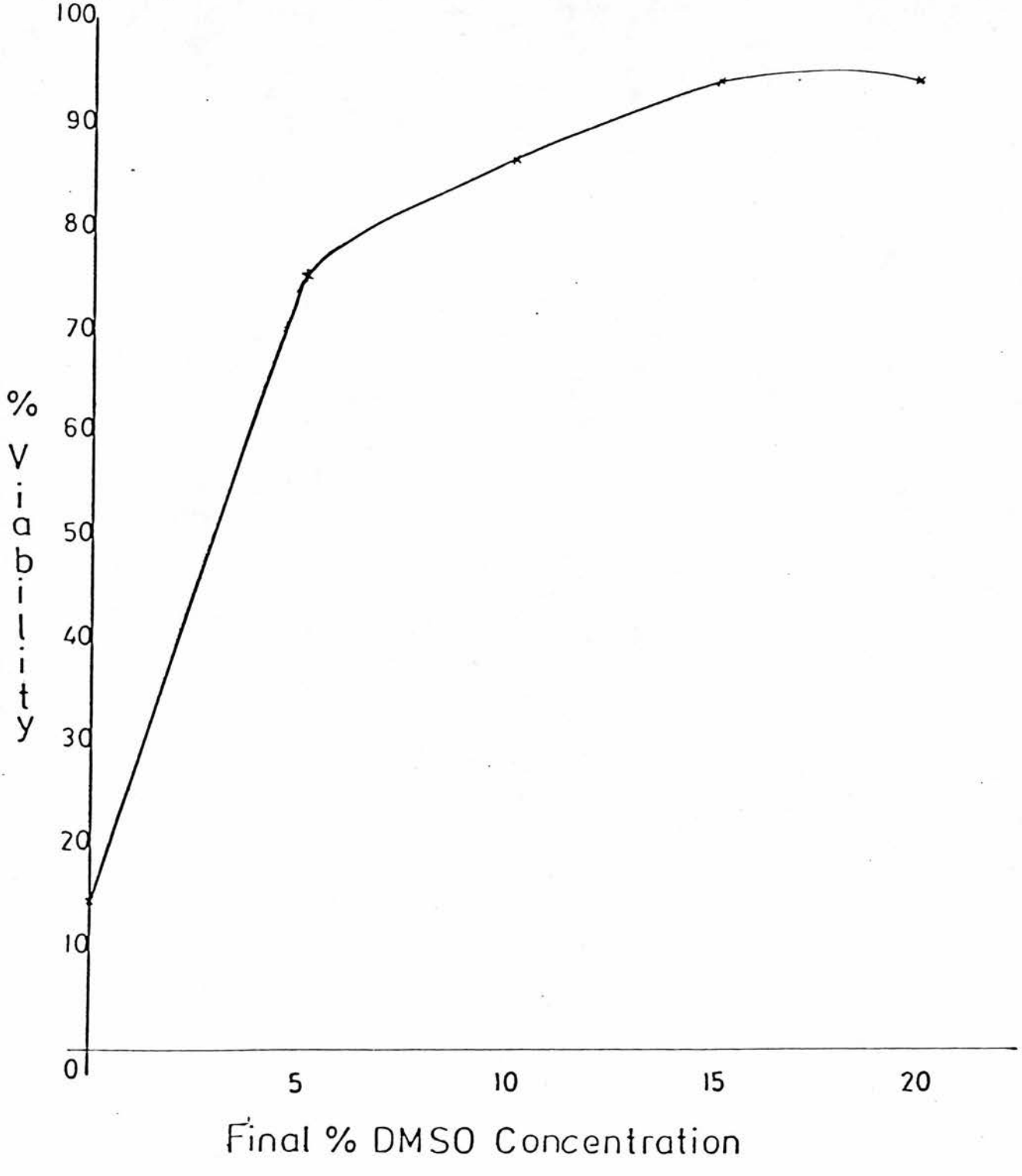


Figure 7

Graph showing the percentage recovery against the final percentage DMSO concentration.

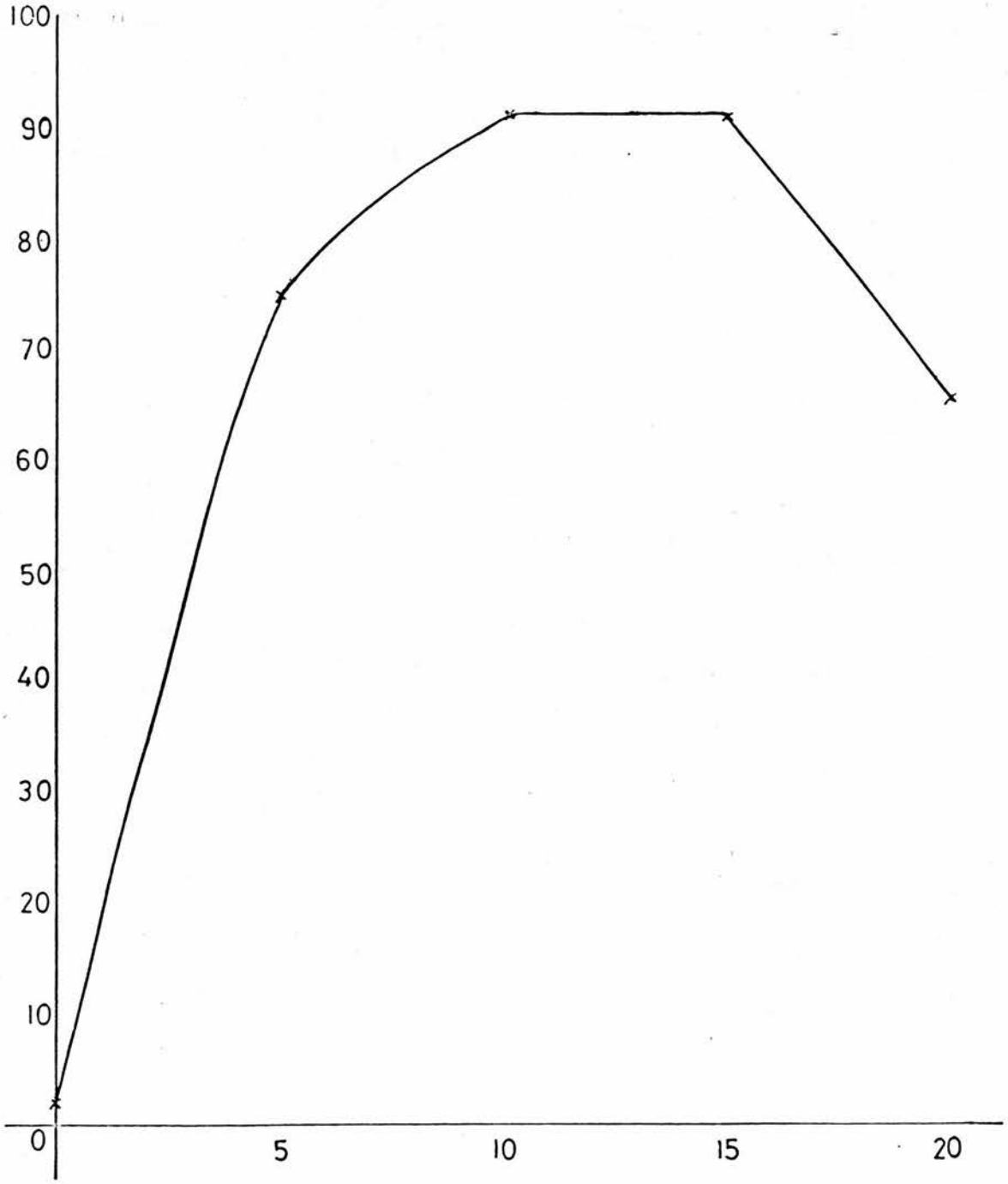


Figure 8

Graph showing the percentage viability against the final percentage DMSO concentration

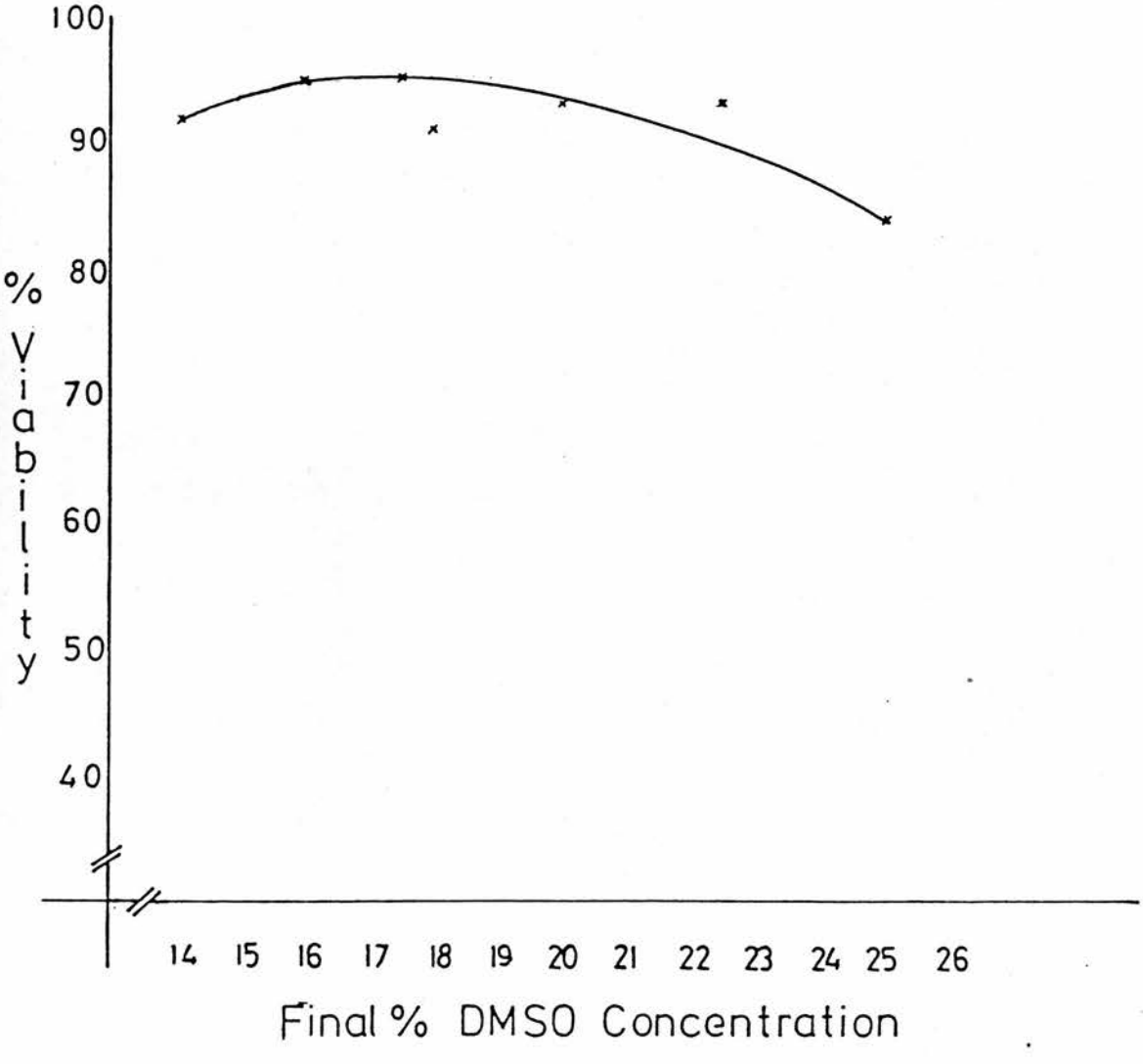
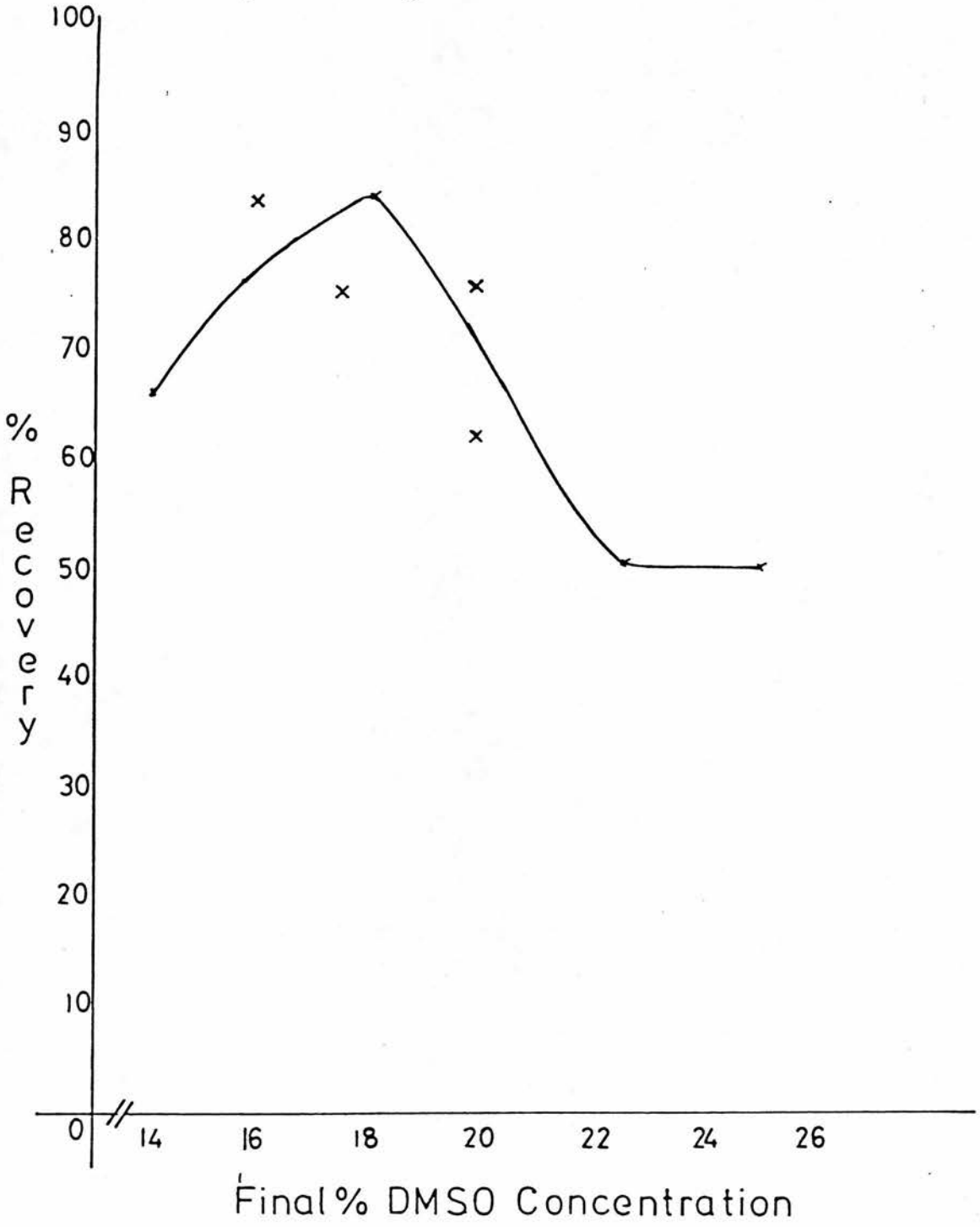


Figure 9

Graph showing percentage recovery against final percentage DMSO concentration



9. Recovery of Lymphocytes from Whole Blood Before and After Freezing.

The purpose of this experiment was to determine the yield and recovery of lymphocytes from 12 mls. of whole blood using the routine separation techniques described above.

Lymphocytes from ten Welsh Mountain sheep were prepared and frozen in the normal manner. Additionally, differential white blood cell and total white blood cell determinations were made on the whole blood preparations. The methods for total white blood cell counts and differential white blood cell counts are described in Chapter 3. 12 mls. of whole blood was used for the preparation of the lymphocyte suspension. These values allowed the calculation of the recovery of lymphocytes from whole blood. Routine procedures for freezing were followed. These results are presented in Table 14.

In all ten preparations, the purity of the prepared cell suspension prior to freezing was greater than 98% and the viability was greater than 99%. The total white blood cell counts are in agreement with those reported by Schalm (1965) and M. H. Blunt (1975), (i.e. 2 to 9 x 10^7 lymphocytes/ml.). These same authors however, report that the percentage of lymphocytes varies between 40% and 75%. The values reported here fall outwith this range. The high values may be caused by the use of heparinised blood for smears. Heparinised blood was used so that the same sample that was used for the total white blood cell count and the lymphocyte preparation would also provide the blood smear. The low value (37%) for cell 3R296 may be explained otherwise. This animal had marked eosinophilia (16% eosinophils).

Table 14

The Recovery of Lymphocytes from Whole Blood Before and After Freezing

Sheep Identity	Whole Blood				Cell Preparation		After Freezing		
	Total Volume of Whole Blood Used (mls.)	Total White Blood Cells per ml. Whole Blood x 10^{-6}	Lymphocytes as a Percentage of the Total White Blood Cells	Lymphocytes per ml. Whole Blood x 10^{-6}	Lymphocytes per ml. Cell Preparation x 10^{-6}	Percentage Recovery of Lympho-cytes from Whole Blood	Lymphocytes recovered after Freezing and Thawing per ml. cell Suspension x 10^{-6}	Percentage Recovery of Lympho-cytes from cell Preparation	Percentage Recovery of Lymphc-cytes frcm Whole Blood
3R045	12	70.2	42	29.5	7.5	2.1	1.1	54	1.1
3R091	12	27.6	82	22.6	8.2	3.0	1.6	54	1.6
3R153	12	21.8	82	17.9	10.4	4.8	2.5	43	2.1
3R217	12	44.8	42	18.8	7.9	3.5	2.1	59	2.1
3R230	12	28.6	93	26.6	14.3	4.5	2.2	49	2.2
3R296	12	31.4	37	11.6	15.1	10.8	5.1	47	5.1
3R318	12	20.4	73	14.9	5.9	3.3	1.4	42	1.4
3R488	12	45.0	66	29.7	4.6	1.3	7.5	58	7.5
3R579	12	29.8	78	23.2	4.9	1.8	1.0	57	1.0
3R616	12	27.2	56	15.2	12.4	6.8	4.6	67	4.6
Mean	-	-	-	-	-	4.2	-	53	2.9

Eosinophilia in humans can be caused by a heavy gastro-intestinal parasite burden (Butterworth, 1977). Due to the type of husbandry employed, heavy worm burdens are not uncommon in sheep. To the best of my knowledge, eosinophilia due to gastro-intestinal parasites has not been shown in sheep. It may, however, be implied by analogy. This eosinophilia could cause a reduction in the proportion of lymphocytes in whole blood.

The total recoveries before freezing vary between 1.3% and 10.8%. These recoveries, while low, are a necessary by-product of the methods employed for isolating pure lymphocyte populations. This matter is more fully dealt with in the discussion after cytotoxicity testing in Chapter 3.

10. The Use of Cryopreserved Cells in Cytotoxicity Testing

Before cell suspensions can be used for cytotoxicity testing, they must be reasonably pure suspensions of reasonably viable cells. For work undertaken for this thesis, minimum standards of 95% purity and 95% viability were set. Cryopreserved cell suspensions prepared using the procedures outlined above nearly always met these criteria. With these improved procedures, there was no discernible increase in cell death due to non-specific lysis where the cells were tested in microlymphocytotoxicity tests.

However, before the cells were used for routine cytotoxicity tests, it was necessary to ensure that the freezing process produced no relevant permanent changes in the sheep lymphocytes. The relevant changes are those which affect the expression of the antigens of the thawed lymphocyte population. Such changes could be of two types.

They could be non-specific. The lymphocytes from all sheep could become more or less sensitive to antibody mediated complement dependent cytotoxicity. For example, a possible change could affect specific lymphocyte subclasses causing an increased or decreased sensitivity to antibody. This change would be non-specific with respect to specific antigens

Such changes could also be specific. They would therefore result in the selective depletion or addition of specific antigens. If these changes occurred they could possibly reflect different modes of membrane attachment amongst the antigen-carrying molecules. Consequently, different antigens would have differential susceptibilities to the freeze-thaw process.

In order to check that these events did not in fact take place, two experiments were carried out. The first of these was designed to detect any changes in sensitivity of frozen cells. The second experiment was designed to detect any changes in antigen expression. These experiments are now discussed separately.

a) <u>The Effect of Freezing and Thawing on the Sensitivity of Sheep Lymphocytes to Lysis in a Type II Microlymphocytotoxicity Test</u>

Nine sheep were each tested against 20 positive sera of varying titre. In order to ensure that each cell was positive for all of the 20 sera, different groups of sera were used for each animal. Although several sera were common to several animals. Each serum was titrated from neat to 1/16 in doubling dilutions. Lymphocytes from each animal were tested both fresh and frozen in a type II microlymphocytoxicity test (described in Chapter 3).

The fresh cells and the frozen cells were all prepared from the same blood suspension. All nine fresh cells were tested simultaneously under identical conditions. All nine frozen cells when tested subsequently were also treated identically and tested simultaneously.

The cumulative scores for the nine animals tested fresh and frozen, are below in Table 15. Reactions are scored as '4', '3', '2', '1' or '0' according to the percentage of lymphocytes killed (Chapter 3). The cumulative score sums the reactions over each doubling dilution. Thus, an animal which gave '4' reactions with undiluted serum and a '2' reaction at a dilution of 1/2 would have a cumulative score of 6. In this test, cumulative scores have been summed over 20 titrations.

The cumulative score reflects differences in end-points. End points can vary quite considerably even in rigidly controlled tests. Yet, even so, there is very little difference in serum titres when these are tested against fresh and frozen cells. (A mean difference of 4.0 for each serum would represent just one doubling dilution difference per titration.) As the difference for each animal is negligible, it is reasonable to conclude that fresh and frozen lymphocytes do not differ in sensitivity in a type II microlymphocytotoxicity test.

b) The Effect of Freezing and Thawing on Specific Lymphocyte Antigens

No specific experiment was set up to test if cryopreservation can result in the loss or gain of specific lymphocyte antigens. However, a number of independent experiments support the assumption that cryopreservation has no effect on the expression of different lymphocyte antigens.

Table 15

Comparison of Serum Titres against Fresh and Frozen Cells

Sheep Identity	Cumulative Sum of Scores for 20 sera		Mean Difference
	Fresh Cells	Frozen Cells	
582	120	133	+0.65
925	179	166	-0.70
095	166	179	+0.65
286	147	132	-0.75
3G77	170	166	-0.20
4G105	140	147	+0.35
4FD3	113	126	+0.65
6BL2	96	106	+0.50
5S23	135	154	+0.95

Firstly, the previous experiment showed that frozen and fresh lymphocytes react with the same sera and give the same titres. The specificity of the sera was not known, but a number of different specificities would most probably have been represented in the different sera. The absence of any effect in any titration suggests that all specificities tested were unaffected by freezing and thawing. As a large number of tests were carried out, most specificities were probably tested.

Secondly, all lymphocyte antigens so far detected on fresh cells have also been found on cells which had been frozen. A total of seventeen lymphocyte antigens have been found in this study. A description of how specificities were detected is given in Chapter 5. All seventeen antigens have been found on lymphocytes which had been cryopreserved. This suggests that freezing and thawing does not cause the specific loss of any lymphocyte antigen. However, this observation does not exclude the possibility that specific lymphocyte antigens are only occasionally lost.

Thirdly, family studies showed regular inheritance which again suggests that the expression of lymphocyte antigens is unaffected by freezing. Lymphocytes from five rams were tested after freezing and thawing, while lymphocytes from the ewes and their lambs were tested fresh. These families are all Damline families. They are described in greater detail in Chapter 6 (families 5, 6, 7, 8 and 9). After allowing for parentage errors, all specificities present in the offspring but absent in the mothers were also present in the ram. Also, all specificities present in the ram were passed on in a regular mendelian fashion to the offspring. This suggests that freezing is not losing or uncovering extra specificities.

Fourthly, specificities were determined on both fresh and frozen cells for nine different animals. A total of twenty-six specificities were detected on fresh cells. There was one discrepancy. Specificity SP10 was absent on the frozen cells of one sheep. Four other sheep were also positive for SP10. SP10 appeared on both fresh and frozen cells in these four sheep. As specificity SP10 is a poorly defined specificity, the discrepancy between fresh and frozen cells could be a serological error. On repeat testing frozen cells from the same animal, the animal was retyped as SP10 positive. This confirmed that the original discrepancy was serological. However, this experiment cannot be regarded as conclusive because:-

i) the system is still in the process of definition. Several of the defined specificities may actually be mixtures of antigens.

ii) the sample size is small. Only nine animals were tested

iii) some specificities were not tested. The nine animals were only positive for seven different specificities. The specificities present were SP3A (3 animals), SP4A (3 animals), SP6 (2 animals), SP7 (3 animals), SP9 (9 animals), SP10 (5 animals) and SP13 (1 animal).

Finally, when the specificities were defined on the nine fresh and frozen cells, the reproducibility of cytotoxicity testing on frozen cells was also estimated. A total of 1,076 repeat tests were made. There were thirteen discrepancies, giving a reproducibility estimate of 98.8%. This estimate is similar to the reproducibility obtained when fresh cells are tested twice (Chapter 3). This observation also supports the assumption that frozen cells carry identical antigens to fresh cells.

In summary, the results reported here suggest that cryopreservation of sheep lymphocytes has no effect on the expression of lymphocyte antigens. There is no evidence for any loss or gain of specific lymphocyte antigens.

Discussion

Luyet and Keane (1955) were the first to show that there is a zone of subzero temperature, exposure to which can protect cells against damage caused by both rapid cooling and rapid thawing. Luyet and Keane used bull spermatozoa but the same observation was repeated on skin cells from chickens (Taylor, 1960), and on human lymphocytes, human lymphoid cell lines and Chinese hamster fibroblasts by Farrant et al. (1974). The procedure described is both simple and reliable. Therefore the procedure was used in this thesis.

In this chapter, the effect of altering several parameters was studied. Parameters which had an effect included the rate of addition of DMSO, the lymphocyte concentration, the volume of serum in the thawing media and the DMSO concentration. With some parameters only slight effects were observed. This may be because they were without real effect or because other uncontrolled effects were influencing the experiments. This is particularly likely with the first parameters to be investigated. When altering a parameter gave only slight or negligible effects, the procedure adopted was to employ the technique favoured by other investigators.

One unexpected feature of this investigation was the very high (17%) concentration of DMSO necessary. Farrant et al. (1974) reported that 5% DMSO was preferable to 10% DMSO with human lymphocytes. It is difficult to understand why the requirements of sheep and human lymphocytes should be so very different. Mazur (1976), has reviewed the mechanisms of injury and protection in both slow and rapid freezing procedures. He states that additives such as DMSO, glycerol/

glycerol and sucrose only have an effect with suboptimal cooling rates. They act by affecting the flow of water to the external medium during freezing. However, when cells are held at a sub-zero temperature prior to freezing, it is not known if the same mechanisms operate to prevent injury. Further, at a temperature of -26°C. the majority of intra and extracellular water is probably in the form of ice. Therefore, water flow will be greatly restricted. The purpose of this series of experiments was only to develop a suitable technique for cryopreservation of lymphocytes and not to look at mechanisms of injury and protection in cell freezing. Thus, this point was not investigated further.

In summary, this chapter describes the development of a suitable test for the cryopreservation of sheep lymphocytes prior to cytotoxicity testing. The methods used for cytotoxicity testing are described in the next chapter.

CHAPTER 3

CYTOTOXICITY TESTING

Introduction

A. Type I Microlymphocytotoxicity Test

Materials and Methods

Results

1. Preparation of Cell Suspensions
2. Choice of Test Trays
3. Use of Complement
4. The Effect of Adding Eosin Dye
5. Choice of Test Panel
6. Effect of Serum Batch
7. Reproducibility
8. Titrations

B. Type II Microlymphocytotoxicity Test

1. Preparation of Lymphocytes
2. The Effect of Complement Dose and of Incubation Temperature.
3. Choice of Incubation Time
4. Carry-over Effects
5. The Type II Microlymphocytotoxicity Test
6. Reproducibility

Discussion

Introduction

A large variety of tests have been used to detect anti-lymphocyte antibodies. These include complement independent tests such as agglutination (Dausset and Nenna, 1952), capillary migration (Thompson et al., 1968), mixed agglutination (Abeyounis et al., 1964), inhibition of mixed agglutination (Metzgar, 1965), the direct anti-globulin consumption test (Dausset et al., 1961). Complement dependent tests include the complement fixation test (Milgrom et al., 1957). Micro-complement fixation tests have also been developed by several investigators (see e.g. Histocompatibility Testing, 1970). Immune adherence techniques have also been used (Sell, 1970). Undoubtedly the most common test, however, is the microlymphocyto-toxicity test (Mlct.) (Terasaki and McClelland, 1964). Modifications of this test exist (e.g. Kissmeyer-Nielsen and Kjerbye, 1967; Mittal et al., 1968).

Four different methods exist for assessing cell viability. These are (i) phase-contrast microscopy (Terasaki and McClelland, 1964); (ii) dye exclusion, usually either trypan blue (Walford et al., 1965) or eosin Y (Terasaki et al., 1967), dye-exclusion tests are usually read under phase contrast (Joysey, 1969); (iii) fluorochromasia (Bodmer et al., 1967) and (iv) chromium-51 release (Sanderson, 1967; Rogentine, 1967).

In this study, a microlymphocytotoxicity test was used as it is reported to give greater reproducibility than agglutination tests, greater sensitivity than complement fixation tests and is more amenable to large-scale testing than the antiglobulin tests. Dye-exclusion/

exclusion under phase-contrast microscopy was chosen to assess cytotoxicity because this system is both simple and extremely accurate. Eosin was used throughout. Trypan Blue was not used because it binds to serum albumin (Engelfriet and Britten, 1966), and this binding could cause dead cells to remain unstained.

Two different variants of a microlymphocytotoxicity test were used in this study. In our laboratory, we use a microlymphocytotoxicity test for routinely typing cattle lymphocytes (see R. L. Spooner et al., 1978 for a description). This chapter describes the work undertaken by me in adapting this technique for sheep lymphocytes. This modified form of microlymphocytotoxicity test I have called the type I mlct. This test was used for screening sera for positive reactions. The results of the screening are given in Chapter 4.

After screening, a selection of positive sera were titrated. Many of the titres in the test were too low to be useful and it was decided to vary certain test conditions in an attempt to increase the sensitivity of the test. After testing, several parameters of the test were changed. I have called this revised test the type II mlct.

I have first listed the procedure followed in the type I mlct. Then I have discussed the parameters which were investigated in order to adapt the test for use with sheep lymphocytes. The type II mlct. is then described.

A. Type I Microlymphocytotoxicity Test

Methods and Materials

Ficoll-Hypaque: A 9% Ficoll (Pharmacia, Uppsala, Sweden) solution was added to a 38% Hypaque Sodium (Sodium Diatrizoate, Winthrop, Surbiton-upon-Thames, England) solution at room temperature to give a final specific gravity of 1.069. The solution was autoclaved before use and stored at 4°C. in the dark.

Washing Solution: Hank's Balanced Salt Solution (HBSS) (Gibco Biocult, Scotland) was used. Work with sheep lymphocytes had shown that sodium bicarbonate (Gibco Biocult, Scotland) and Hepes (4-(2 hydroxy-ethyl)-1 piperazine ethane sulphonic acids) (Wellcome, Beckenham, England) buffers were unsatisfactory (discussed later). Therefore a pool of heat-inactivated non-reactive sheep serum (NRSS) drawn from six to ten rams was used, at a final concentration of 10%, as a buffer. All rams in the pool were previously screened for cytotoxicity. All rams thus screened were negative. The pH of serum buffered HBSS was always 7.3 ± 0.1.

Microtitre Plates: Terasaki microtest plates were used. Sera and serum controls were plated out under liquid paraffin before use. These were then stored frozen at -25°C. Plates were thawed at 37°C. for twenty minutes before use. Each plate was thawed only once. On removal from the freezer, all wells were checked to ensure the presence of a frozen serum droplet.

Complement: Unabsorbed, pooled rabbit serum was used as a source of complement. The pool was stored frozen in aliquots in liquid nitrogen until needed. When needed, aliquots were thawed, filtered, refrozen/

refrozen and kept in small quantities at -25°C. Each sample was thawed only twice. The same batch of complement was used for all type I microlymphocytotoxicity tests.

Eosin: A 5% stock (Eosin yellow shade B.D.H., Poole, England) solution in distilled water was kept at 4°C. Just before use, 1 ml. of this 5% solution was added to 2 mls. Hank's double strength balanced salt solution with 20% NRSS and 1 ml. distilled water.

Fixative: A 40% formaldehyde solution in water (i.e. formalin Hopkin and Williams, Chadwell Heath, England) was diluted 2:3 with phosphate buffered saline (PBS) (pH 7.2) then neutralised 95:5 with 0.15 m. disodium hydrogen phosphate. This was then ready for use and was stored at 4°C.

Phosphate Buffered Saline: Phosphate Buffer was made by mixing 0.2M. disodium hydrogen orthophosphate with 0.2M. sodium dihydrogen orthophosphate until pH 7.2 was reached. 1 part phosphate buffer was then mixed with 8 parts 0.9% saline. The conductivity was then measured and, if necessary, adjusted to 14-15 with distilled water.

Animals: All animals used came from farms of the Animal Breeding Research Organisation. The source of serum samples is in Chapter 4. A panel of 40 sheep was used to provide lymphocyte preparations for screening each serum sample. These 40 sheep were eight sheep from each of five breeds (Finnish Landrace x Dorset Horn, Cheviot, Border Leicester, Southdown and Welsh Mountain). These five breeds were carefully chosen as breeds which would not be expected to be inbred. The sheep were chosen to be as unrelated as possible.

Titrations: All dilutions were made with a pool of NRSS. All titration experiments used doubling dilutions.

Serum: Sheep were bled by jugular venepuncture into dry evacuated glass vessels. For small samples, siliconed evacuated 7 ml. (Becton-Dickinson Vacutainers, U.S.A.) tubes were used as they produced less haemolysis in the serum samples than non-siliconed glassware. Blood was left for 24 hours at room temperature to allow the clot to retract. The sera were then poured off and centrifuged at 1,500 g. for 20 minutes to clear. Sera were then subdivided into small quantities and stored at -25°C. All sera were heat inactivated at 56°C. for 30 minutes before testing.

Plasma: Sheep were bled by jugular venepuncture into 7 ml. evacuated glass tubes containing lithium heparin to give a final concentration of 20-40 I.U./ml. whole blood (Becton-Dickinson Vacutainers, U.S.A.). After 1 ml. of blood had been removed for preparation of lymphocytes, the whole blood was centrifuged at 1,500 g. for 20 minutes and the plasma/

plasma removed and stored frozen at -25°C. Plasma samples were subsequently treated similarly to serum samples.

Preparation of lymphocyte suspensions: 0.95 ml. of heparinised blood was layered carefully onto 2.5 mls. Ficoll-Hypaque. This was centrifuged at 1,500 g. for 20 minutes. The lymphocyte rich interface was then removed and suspended in HBSS buffered with 10% NRSS. The cells were washed twice at 100 g. for 15 minutes to remove platelets and dead cells. The cell pellet was then resuspended in 1 ml. HBSS + 10% NRSS. Cell number, viability, granulocyte and erythrocyte contamination was then assessed under phase contrast microscopy. Cell concentrations were then adjusted where necessary to 1.0-2.0 x 10^6 lymphocytes/ml. Using non-reactive serum as a buffer, viability was always greater than 99% and granulocyte contamination minimal (<5%). Erythrocyte contamination varied from 0-40%, although with the vast majority of sheep cell suspensions, erythrocyte concentration was less than 1%. Cell suspensions containing more than 5% sheep red blood cells were purified by hypotonic lysis in distilled water for 8 seconds. Isotonicity was restored with equal volumes of double-strength HBSS + 20% NRSS. Platelets were not seen.

Cytotoxicity Testing: 1 ul. of cell suspension was added to 1 ul. of serum. After incubation at 37°C. for 30 minutes, 1 ul. of undiluted pooled rabbit serum was added as a complement source. All plates were then incubated at 37°C. Forty minutes after complement addition, 1 ul. of 1.25% eosin in HBSS + 10% NRSS was then added and the plates returned to the incubator at 37°C. 20 minutes after adding the vital dye, 2 ul. of fixative was added to fix the reaction.

Scoring: All plates were read under phrase contrast using a Nikon inverted microscope using binocular x 10 eyepieces and a x 10 objective. Wells were scored thus:

Percentage Lysis:	0-5	6-10	11-40	41-60	61-94	95-99	100
Score:	-	(1)	1	2	3	(4)	4

For most purposes, and unless otherwise stated, scores of 2 or over were treated as positive, anything less as a negative.

Experimental Design: All protocols were arranged to allow all reagents and solutions to be dispensed with a Hamilton multiple dispenser and all cells on one plate to come from the same lymphocyte preparation. All plates included at least two positive and two negative wells. The negative wells also served as a complement control by showing that the pooled rabbit serum was not cytotoxic for the cells being tested.

Each serum sample was tested undiluted and at a dilution of 1/8. Each sample was tested against the 40 panel sheep, and, on occasion, other non-panel animals.

All results obtained from testing one batch of serum samples were recorded on one protocol sheet. This avoided the need for separate protocol sheets for each serum sample and prevented the unnecessary transcription of information to summary sheets. All scores could be transferred directly to the computer.

Results

1. Preparation of Cell Suspensions

One important factor in the accuracy and reproducibility of microlymphocytotoxicity tests is the purity and viability of test cell suspensions. The presence of contaminating platelets, granulocytes and monocytes may lead to false negatives by absorption of antibodies from the sera. The presence of a high background of dead cells can obviously cause reading errors. Additionally, low purity and/or low viability cell preparations are difficult to read, especially in the presence of debris. This too can cause reading errors either directly or through increased fatigue. Factors affecting purity and viability are briefly discussed below.

(i) Separation Procedures

There are a variety of methods for isolating lymphocytes from peripheral blood. These include filtration through cotton wool (Barr and Perry, 1976), sedimentation with plasmagel (Elves, 1976), sedimentation with dextran (Engelfriet and van den Berg-Loonen, 1976) and separation by layering onto a density gradient medium (usually made with Ficoll and Sodium Metrizoate and/or Sodium Diatrizoate) (Boyum, 1968). All these methods have the option of removing phagocytic cells by pre-incubation with carbonyl iron. In most species where lymphocyte typing is used, the use of a density gradient is the method of choice. In sheep, the use of a density gradient medium, composed of 9% aqueous Ficoll solution/

solution mixed with a 38% aqueous solution sodium diatrizoate gave acceptable results and this method was used throughout. Initial trials with either commercial preparations "Lymphoprep" (Nyegaard & Co., Oslo, Norway) or "Ficoll-Paque" (Pharmacia, Uppsala, Sweden) or mixtures made by me to a final specific gravity of 1.077 $\pm$ 0.001 gave unacceptably high contamination with granulocytes and erythrocytes. Following results obtained in cell culture, a specific gravity of 1.073 was used for initial tests. Following a limited trial, a specific gravity of 1.069 was used for the type I mlct. This gave a final granulocyte contamination of less than 3% in over 200 samples taken from twelve different breeds and cross-breeds. Erythrocyte contamination varied from 0-40% with the vast majority of sheep tested having negligible erythrocytes. Interestingly, sheep with high erythrocyte contamination gave consistently high levels in repeat tests. Large numbers of contaminating red blood cells were especially common in sheep from the Tasmanian Merino and Welsh Mountain flocks kept at Dryden Field Laboratory, Roslin, Midlothian. These contaminating cells were removed by hypotonic lysis in distilled water.

(ii) Collection of Samples

One of the major problems in initial tests was cell clumping. In certain samples the anticoagulated blood flowed freely, but after centrifugation a clump of mixed granulocytes, platelets, erythrocytes and lymphocytes settled at the interface. Trials using ACD or EDTA as anticoagulants prevented clumping, but ACD gave a reduced white cell yield. EDTA-blood needed heparin to prevent clotting when calcium was added in the washing stage. A small test using lymphocytes prepared/

prepared identically with EDTA-blood and heparinised blood on the same sera showed no effect between the two samples. However, the use of EDTA meant that heparin would have to be present in the washing medium. Heparin is known to have an anti-complementary effect. Therefore, EDTA was not used as an anti-coagulant.

Therefore it was decided to use dry lithium heparin as anticoagulant. (This would be diluted out in subsequent washing steps.) Special care was taken to ensure thorough mixing, by multiple inversions of the blood immediately after bleeding. This thorough mixing eliminated the problem of white cell clumping.

(iii) Washing Solutions

In contrast to the results observed with cattle and human lymphocytes, Hank's Balanced Salt Solution was not suitable for washing sheep lymphocytes. This solution gave readily discernible background cytotoxicity varying from 1% to as much as 10% with some animals. This background lysis was reduced, but not abolished, when stoppered test tubes were used. HBSS is buffered by a bicarbonate-carbonate system. Lysis is probably due to an increase in pH as HCO_3^- dissociates to form CO_2 and H_2O. The CO_2 will then escape into the atmosphere.

HBSS buffered with 25 mM Hepes was also tried as a washing solution. In a previous study on sheep leucocytes, Ford (1973) used 0.9% saline as a washing solution. These both proved unsatisfactory. Two washing solutions gave excellent viability (>99%): they were Phosphate Buffered Saline pH 7.2 and HBSS buffered with a pool of non-reactive sheep serum at a final concentration of 10%. Unfortunately, even when centrifuged at the same relative centrifugal force/

force as HBSS + 10% NRSS there was considerable platelet contamination in the PBS washed preparations. The different viscosity of PBS and serum buffered HBSS is probably responsible for the difference in platelet contamination. For this reason, HBSS + 10% NRSS was used as a washing solution throughout these studies.

Incidentally, 0.9% saline buffered with 10% NRSS also gave excellent results but for logistic and aesthetic reasons it was decided to use HBSS + 10% NRSS.

(iv) Cell Storage

Whole sheep blood stored overnight either at 4^{o} or at room temperature gave increased background cell death, which was present even after separation on Ficoll-Hypaque and two washes in HBSS + 10% NRSS. Additionally, cells prepared after overnight storage gave poor reproducibility when compared to samples tested on the same day as bleeding. This poor reproducibility was largely due to an increased frequency of positive reactions when compared to fresh samples

To test the effect of storage on reproducibility, eight Welsh Mountain sheep were bled and tested in a type II mlct. in the normal manner. The next morning the same sample tubes were used to carry out a second set of tests. The age of the blood (Table 1) refers to the time since collection at the start of the cell separation procedures. Each of the eight sheep was tested against 240 serum samples on both occasions. The tests were scored independently of each other and were not cross-checked.

Table 1

Comparison of the Behaviour of Lymphocytes

Prepared from Fresh and Day Old Blood in a Microlymphocytotoxicity Test

		Lymphocytes Prepared from Fresh Blood	
		Number of Positive Reactions	Number of Negative Reactions
Lymphocytes Prepared From Day Old Blood	Number of Positive Reactions	819	173
	Number of Negative Reactions	13	915
		832	1088

992	
928	
1920	

$X^2 = 128.7$ $P < 0.001$

The 2 x 2 table (Table 1) refers to the positive and negative reactions of the 240 sera against the eight animals. All the 240 sera, except four negative controls, contained anti-sheep lymphocyte antibodies, as shown by previous tests. In this test 42 additional sera failed to react with any of these eight sheep.

If fresh blood and one day old blood behaved identically, the discrepant results (+ - and - +) should occur randomly. In Table 1 there are many more discrepant positives in the 22 hour old sample (173 -v- 13). These extra positives may be false positives caused by non-specific lysis. Alternatively, they may reflect a greater sensitivity of the test. It may be that the surviving cells are not as healthy in day old blood and more susceptible to lysis. This greater vulnerability coupled with higher backgrounds could account for the results above.

2. Choice of Test Trays

Three different types of test tray were tried, plastic Falcon (3034), plastic Nunc test trays and glass plates prepared in cut-out Falcon beds in our laboratory.

In plastic plates, optical interference, especially at the periphery of the wells, can be quite considerable. This problem was not seen when flat glass was used as a bed for the test solutions. Unfortunately, it was difficult to prevent wells mixing in a small proportion of tests. A study of reproducibilities showed 49 discrepancies in 2,004 repeat tests when plastic Falcon plates were used (97.6%) and 48 discrepancies in 1,800 repeat tests on glass plates (97.3%). However, more tests were/

were unreadable on glass plates because of wells mixing. For this reason glass plates were not used in subsequent tests. Of the two plastic plates, Falcon plates appeared to give less optical interference. This was especially true when small amounts of liquid paraffin were used to cover plates before plating out sera. After dropping out sera, excess liquid paraffin was added to present evaporation. Falcon plastic plates were used for all tests subsequently carried out.

3. Use of Complement

One of the possible sources of variation in a cytotoxicity test is complement. Many authors have stressed that it is important to standardise the complement used for testing on different occasions and also to determine the most suitable use to be made of complement.

With respect to standardising the complement used, intra-batch variation is minimised by long-term storage at -196°C., minimal freezing and thawing, not keeping complement frozen at -20°C. for longer than necessary (and in any event not longer than five weeks) and finally, not leaving complement at room temperature for longer than necessary. Inter-batch variation can also be reduced by using pooled rabbit serum and by comparing different batches. Where batches differ in sensitivity, the incubation time can be adjusted to allow for this.

It is well known that the effectiveness of complement in cytotoxic and haemolytic tests depends on which species acts a complement source and the species from which the antibody is derived (Coombs et al., 1961). Rabbit serum was used as a complement source because a previous trial, with/

with sheep anti-sheep lymphocyte sera, by Ford (1973) had shown this to be the most effective when sera from humans, guinea pigs, sheep and rabbits were tested. Rabbit serum is also the most common choice for a complement source in other species. Although, the use of rabbit serum is not universal. For example, guinea pig serum is more effective than rabbit serum with goat anti-lymphocyte alloantisera (van Dam et al., 1976).

In one-stage tests, a source of antibody and a complement source are added simultaneously to the cell suspension. In two-stage tests, the complement source is added after the antibody. Two-stage tests have been shown to be more sensitive in humans (Ahrons and Kissmeyer-Nielsen, 1973) and in dogs (Smid-Mercx et al., 1975). Two-stage tests were used throughout the study.

A 30 minute interval was allowed between the addition of antibody and the addition of complement. This is in accordance with standard procedures in the human.

4. The Effect of Adding Eosin Dye

Eosin dye can be added to cell suspensions to assess the level of cell death (Hanks and Wallace, 1958). Eosin is a vital dye. Initially, the dye penetrates both live and dead cells. After a short interval living cells exclude the dye and under phase contrast appear bright and shining. Dead cells do not exclude the dye and appear dull and dark.

In cytotoxicity tests, the interval between adding eosin dye and adding fixative is usually 1-5 minutes (e.g. Mittal et al., 1968). In my tests, I chose to add eosin dye fully 20 minutes before adding fixative./

fixative. A twenty minute interval allowed me to handle a large number of tests more easily. Eosin dye could be added to all plates before any fixative was added.

It is possible that eosin dye, at relatively long exposure times, might, itself, be harmful to sheep lymphocytes. Hanks and Wallace (1958) suggested that 0.45% eosin can have a deleterious effect on mouse fibrocytes after 30 minutes incubation. In order to test this possibility, I compared the frequency of cytotoxic reactions and also the serum titres, both in the presence and in the absence of eosin dye.

To assess the effect on serum titre, the same lymphocyte suspension was tested twice against 69 sera. Each serum was tested at six doubling dilutions, which were chosen to include the end-point of the serum. The two replicates were treated identically, except that eosin was added twenty minutes before the fixative to one replicate. No dye was added to the other replicate. Cytotoxicity was assessed by phase contrast microscopy alone.

Twenty-four of the sixty-nine sera were positive in the test. The other forty-five sera were negative. Twenty-two of the twenty-four sera gave identical reactions at all the dilutions tested. The other two sera differed slightly at their end-points. In both cases the replicate with eosin gave a '2' reaction (41-60% cell death), while the replicate without dye gave a '1' reaction (11-40%). I do not feel that the two discrepant reactions are significant. Incubation with eosin dye for twenty minutes appears to have no effect on serum titre.

The effect of incubation with eosin on the frequency of cytotoxic reactions was also assessed. This test was carried out simultaneously with the test which looked at serum titres. In addition to the sixty-nine end-point determinations described above, the same lymphocyte suspension was also tested against sixty-six different serum samples. The serum samples were undiluted. There was no difference in the reactions of the two replicates. Both replicates gave positive reactions with ten sera and negative reactions against the other fifty-six sera. This test suggested that eosin incubation had no effect on the frequency of positive reactions in a micro-lymphocytotoxicity test.

The two tests show that a twenty minute incubation with eosin does not affect test sensitivity or reproducibility but does simplify reading.

Choice of Test Panel

The most efficient size of test panel will be determined by the frequency of the antigen. In sheep at the time of starting this study, the frequency of lymphocyte antigens was not known. Information from other species suggested that there were a variety of lymphocyte antigens present in all mammalian species. Secondly, certain antigens had a very low frequency. Consequently in screening sera, there is a real possibility that positive sera could be scored as negative simply because the test panel did not contain antigens with which the serum would react. This is especially true for sera which react with a low percentage of sheep. It is, of course, precisely these sera which are likely to prove most useful. For

different/

different sizes of test panel (n) the probability (p) of detecting antisera reacting at a given frequency (f) is given by $p = 1-(1-f)^n$ (Sales, 1977 - personal communication). Forty sheep were available for serum screening. A panel of forty animals implies that 95% of antisera with a phenotypic frequency of 7.5% will react with at least one animal out of forty.

The choice of breeds is also important in sheep. Over fifty sheep breeds are maintained in the British Isles. The breeds are generally kept by breeding with other animals of the same breed. Although extensive gene flow occurs, especially between similar breeds. Nonetheless, in several breeds, the level of polymorphism is likely to be restricted due to founder effects and also to population bottlenecks. In order to provide heterogeneity, sheep were chosen from a wide variety of breeds which had not as far as is known suffered population bottlenecks or founder effects.

The breeds chosen were Southdown, Cheviot, Border Leicester, Welsh Mountain and a cross-bred flock (Finnish Landrace x Dorset Horn). Eight sheep, as unrelated as possible, were chosen from each of the four breeds and the one cross-bred flock. The structure of the panel varied with time as animals were culled or died from natural causes. In certain instances, replacement animals were related to surviving sheep on the panel.

6. Effect of Serum Batch

Hirata and Terasaki (1972) have reported that autologous or allogeneic serum can inhibit the cytotoxicity of anti-HLA sera on human lymphocytes. However,/

However, they also reported that "the cell masking substance lacks specificity as regards anti HL-A specificity and also HL-A specificity of target lymphocytes". As long as the effect of serum is consistent through all tests, the use of serum should not affect the determination of antigen specificities.

In humans, HLA antigens have been found in serum (van Rood et al., 1970; Charlton and Zmijewski, 1970). The HLA antigens are present at low concentrations. It is possible that the low concentrations of serum antigens may have a specific effect on sera of certain specificities, perhaps by binding to specific antibody and preventing cell lysis. This possibility could not be excluded at this stage as the specificity of the antisera was not known.

However, the concentration of serum in the washing solution was only 10%. Further, 6-10 different unrelated rams were used to provide a serum pool. Therefore, the concentration of any antigens present in the final mixture would be very low indeed.

A number of tests were carried out with two serum batches to exclude any gross effect of serum batch. Two lymphocyte preparations were made from each of ten sheep. The preparations were made identically, except that two different washing solutions were used. One washing solution was buffered with serum batch A. The other washing solution was buffered with serum batch B. Each of the twenty lymphocyte preparations was then tested simultaneously against 120 sheep antisera. There were sixteen discrepant reactions amongst the 1,200 duplicate tests. The sixteen discrepant reactions appeared to be randomly distributed amongst the ten sheep.

The reproducibility estimate (98.7%) obtained here is very similar to a value found in a reproducibility experiment (quoted later) which was 97.6%.

The absence of any apparent specific effect of different batches of serum, and the high reproducibility between different batches suggested that the serum batch does not affect the specificity of lymphocytotoxic reactions.

7. Reproducibility

2,004 repeat tests were carried out, 49 were discrepant. The reproducibility estimate is 97.6%. Additionally, in screening sera against the panel, one to five animals were tested twice against test trays containing identical batteries of sera. All these small scale (120 - 1,200 repeat tests) reproducibility tests conducted on repeat tests gave greater than 95% reproducibility. It ought, however, to be added that these small scale tests had a high frequency of negative reactions (usually 80-90%). The repeat tests were carried out in the same fortnight and utilised an identical plate layout.

Further, 6,000 tests were re-read in an attempt to find the reading error rate. Only three tests were read differently. This result suggests that reading is unlikely to be a major source of error.

8. Titrations

Thirty four sera were titrated. All dilutions were doubling dilutions. The results are given in Table 2. All sera came from/

Table 2

Titres of 34 Sheep Sera in a Type 1 Mlct.

Serum	Positive Animals	Negative Animals	Total Sheep Tested	Maximum Titre
3E16	13	5	18	1/4
5Z4	7	2	9	1/4
4E40	2	2	4	1/2
1N090	16	2	18	1/8
1N180	4	3	10	1/8
1N185	7	4	12	1/2
1T122	3	5	9	1/16
1T239	13	2	17	1/4
1T356	3	5	9	1/8
1T465	2	4	6	1/4
2N045	1	7	8	1
2N142	6	2	8	1/8
2N212	1	6	8	1
2N223	3	6	9	≥1/32
2T061	11	12	24	≥1/32
2T075	7	3	10	1/8
2T156	7	6	13	1/4
2T229	18	1	20	1/4
2T238	2	5	7	1/4
2T275	2	10	12	1
2T277	5	2	7	1/2
2T278	3	5	8	1/8
2T315	5	4	9	1/4
2T389	5	5	10	1/8
64363	5	1	6	1/4
64486	9	4	13	1/4
64444	7	0	7	1/8
64465	5	0	5	1/8
64489	2	0	2	1/2
286	3	3	6	1/2
3G77	3	7	10	1/2
3W26	2	2	4	1/4
83C02	6	2	8	1/4
3W4	5	2	7	1/8

from parous sheep. All sera were titrated against all positive members of the panel of forty sheep. The sera were also titrated against negative sheep as controls. Despite the fact that several sera were selected as 'strong' sera, many of the titres are very low.

B. Type II Microlymphocytotoxicity Tests

As the majority of the titres in the type I mlct. were too low to be useful for cytotoxicity testing, it was decided to try and improve the sensitivity of the microlymphocytotoxicity test, by investigating certain parameters of the test. These investigations are described under the headings, preparation of lymphocytes, complement and incubation temperature, incubation time, carry-over effects, the type II microlymphocytotoxicity test and finally reproducibility.

1. (a) Preparation of Lymphocytes

As before, sheep lymphocytes were prepared by centrifugation over a Ficoll-Hypaque suspension followed by two washing steps.

The purpose of these experiments was to determine the optimum specific gravity for separation of sheep lymphocytes independent of Ficoll concentration.

The previous technique for preparation of a density gradient medium (Ficoll-Hypaque) was to add a 38% sodium diatrizoate solution (w/v) to 500 mls. of a 9% Ficoll solution (w/v) until the specific gravity was 1.069. As approximately 150 mls. of sodium diatrizoate proved to/

to be necessary for this, the final concentrations were about 8.7% sodium diatrizoate and 6.9% Ficoll. The standard commercial preparations of Ficall-Hypaque prepared for human white cell separations (Lymphoprep) contain 5.6% Ficoll and 9.6% sodium metrizoate.

I decided to make up a standard calibration curve for Sodium Hypaque concentration and specific gravity at a constant Ficoll concentration. This graph is shown in figure 1. This has been verified for three different batches of Hypaque on two batches of Ficoll.

Using this graph 4 preparations of Ficoll-Hypaque were prepared. Each had a final concentration of 6% Ficoll.

A.	Specific gravity	1.048	5% Sodium Hypaque
B.	Specific gravity	1.064	7.5% Sodium Hypaque
C.	Specific gravity	1.076	10% Sodium Hypaque
D.	Specific gravity	1.090	12.5% Sodium Hypaque

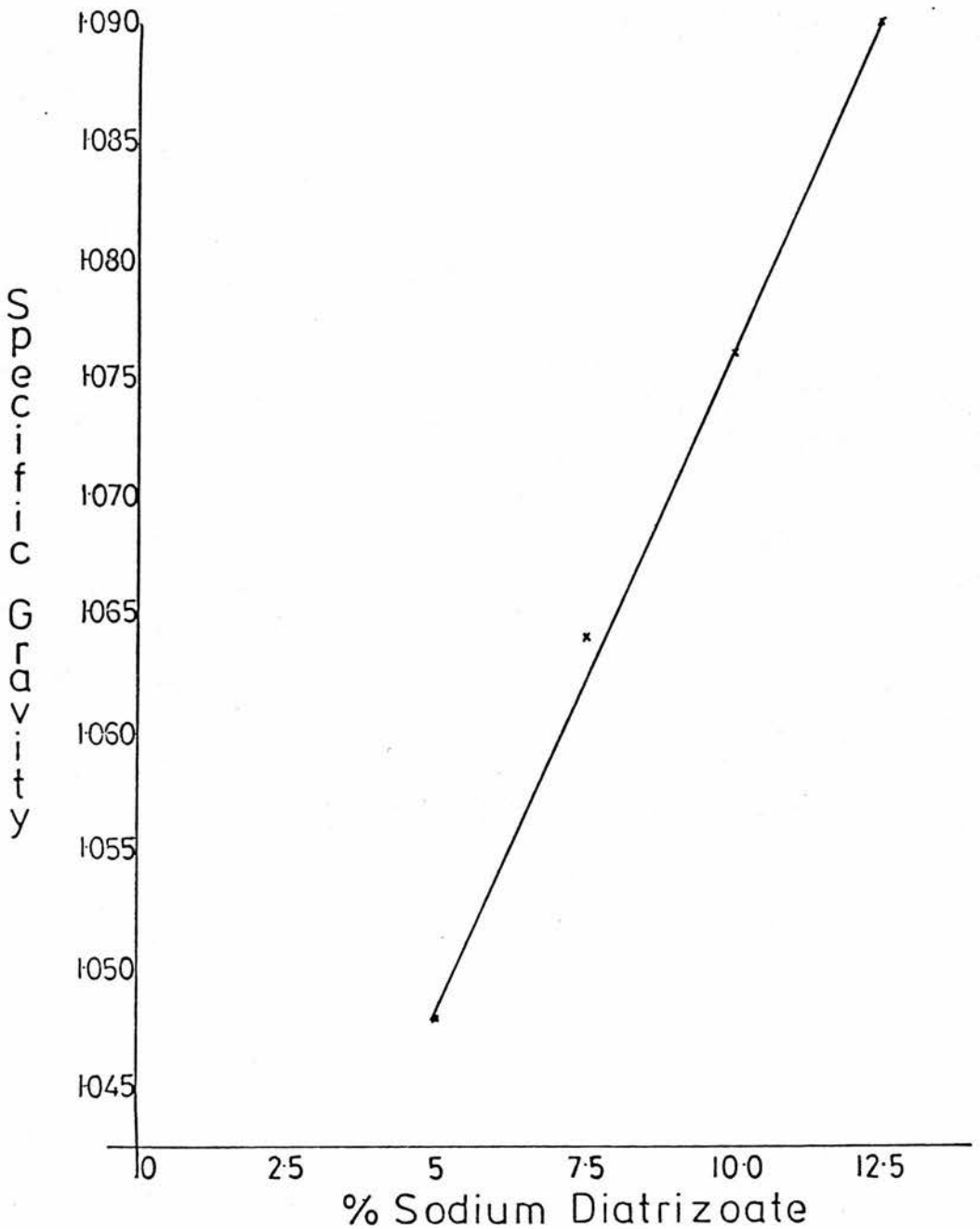
Samples of whole blood in lithium heparin were drawn from four unrelated ewe-lambs of the same age and from the same cross-bred flock (Finnish Landrace x Dorset Horn). 1 ml. of whole blood from each animal was carefully layered onto 2.5 mls. from each of the four preparations. All sixteen samples were spun 20 minutes at 1,500 g. and the appearance of the interface examined visually.

In preparation D there was very little sedimentation of red blood cells. Preparation A was also unacceptable, as here the leukocytes finished below the interface and only just

above/

Figure 1

Calibration Curve of Specific Gravity Against the percentage of Hypaque(Sodium Diatrizoate) in a 6% Ficoll solution



above the red blood cell pellet. As a consequence of this, I decided to look at the behaviour of sheep whole blood on preparations of Ficoll-Hypaque within the narrower range 1.077 to 1.062.

Four more preparations of Ficoll-Hypaque were prepared. Again each had a final Ficoll concentration of 6%.

A.	Specific Gravity	1.077	10.9% Sodium Hypaque
B.	Specific Gravity	1.072	9.8% Sodium Hypaque
C.	Specific Gravity	1.067	8.9% Sodium Hypaque
D.	Specific Gravity	1.062	8.0% Sodium Hypaque

To test the effect of these concentrations on the preparation of sheep lymphocytes, three essentially similar experiments were carried out. In each experiment, four unrelated animals from the same breed (one experiment used Cheviot sheep, the other two, Welsh Mountain) were bled into tubes containing lithium heparin as described earlier. Lymphocyte preparations were made as for normal cytotoxicity tests. These breeds and animals from these breeds were deliberately chosen. They had given a lower purity when cells were prepared from these animals on previous occasions. Lower purity was due to contaminating red blood cells. The results from these three tests are pooled and presented in Table 3. Cell differentiation was made under phase contrast.

In two Welsh Mountain sheep, red cell contamination in preparation D was too high for accurate counting. These two cells have not been included. The counts for ten other cells are summarised in Table 3.

Table 3

Effect of Altering Specific Gravity at a Constant 6% Ficoll Concentration on the Preparation of Sheep Lymphocytes from Whole Blood

Specific Gravity of Ficoll-Hypaque	Cell Recovery		
	Lymphocytes	Granulocytes	Erythrocytes
1.062 (A)	1,115	66	287
1.067 (B)	1,220	63	592
1.072 (C)	1,692	104	1,548
1.077 (D)	919	80	2,163

As it is the relative proportions of cells and not the cell concentrations which are important, the cell counts are given. Also, as the counts were made under phase contrast, the cells scored as lymphocytes may also include other non-lymphocytic mononuclear cells such as monocytes. Additionally, of the 287 erythrocytes counted for preparation A, 107 came from two animals which had normal lymphocyte recoveries. Other experiments were also carried out using the same experimental design as above, i.e. four samples prepared simultaneously on the four preparations above. As fresh blood was not available, cell counts were not made on these preparations. The appearance of the Ficoll-Hypaque and separated blood confirmed the belief that the optimum specific gravity lay between 1.062 and 1.067.

A solution of Ficoll-Hypaque was then prepared at a Ficoll concentration of 6% and at a specific gravity of 1.064. The final concentration of sodium diatrizoate was 7.75% (figure 1).

Heparinised blood was collected from twenty sheep comprising four ewes from each of five breeds. Lymphocyte suspensions were then prepared and counted, using Ficoll-Hypaque with a specific gravity of 1.064. From the results above, using specific gravities of 1.062 and 1.064, it appeared that sufficient cells for cytotoxicity testing would not always be obtained from 1 ml. of blood. Accordingly, 2 mls. was used for this and all subsequent experiments.

Preparations from these twenty animals were all quite adequate for cytotoxicity testing. They all gave adequate recovery and purity. Granulocyte and erythrocyte contamination was minimal. Subsequent tests/

tests with many animals of different breeds produced a small number of preparations with 1-5% erythrocyte contamination and in one case 20% erythrocyte contamination. (This animal was not used for cytotoxicity testing.) Granulocyte contamination was always less than 3% and usually negligible. Certain breeds gave a very low lymphocyte recovery (e.g. Boreray Blackface) although this was still adequate for cytotoxicity testing. Sheep husbandry is such that apparent breed differences may be due to sire effects or to environmental effects. Therefore, while differences in lymphocyte recovery and erythrocyte contamination were noticed, e.g. in Boreray Blackface and Tasmanian Merino breeds, they were not investigated further.

(b) Cell Recovery

For the type II mlct. 2 mls. of whole blood was used. In the preparation of cells for freezing, different arrangements were made. The recovery of cells using those arrangements is discussed in Chapter 2. Here I discuss only the recovery when cells were prepared for cytotoxicity counting. To assess lymphocyte recovery, it is necessary to determine three things:-

1) The initial proportion of lymphocytes in the white cell population in whole blood. This is done by a differential count in a blood smear,
2) The total white cell concentration in whole blood, and
3) The total cell count and the percentage of lymphocytes in the recovered population.

The methods used and the results are presented below.

(i) Differential White Blood Cell Smears

Conventional techniques were employed for the preparation of blood smears (see e.g. Whitby and Britton, 1963). Smears were fixed and stained in Leishman's Stain (George T. Gurr, London, England). A liberal quantity of stain was poured on and left to allow methyl alcohol to fix the cells. After one minute, twice the quantity of distilled water was added to the slide and mixed thoroughly. After twelve minutes the diluted stain was washed off with excess distilled water and the slide was allowed to dry in air. Differential counts were made using a x 100 oil immersion objective. All white cells seen were scored as basophils, neutrophils, eosinophils, monocytes or lymphocytes. No attempt was made to score large and small lymphocytes separately. Although lymphocytes at either end of the size range can be readily distinguished, there is considerable overlap between the two populations. This overlap is also reported by Schalm (1965). All samples were smeared and stained within four hours of collection.

As the presence of heparin is known to influence white cell smears, smears were made in EDTA blood unless otherwise stated. Samples of fresh EDTA blood from ten sheep were smeared and tested to test the accuracy of my technique. All values were in agreement with those reported in the literature (Blunt, 1975; Schalm, 1965). Lymphocytes varied between 40-75% of total white blood cells.

(ii) Total White Blood Cell Counts

A white blood cell diluting fluid made up with 1.5 mls. glacial acetic acid and 1.0 ml. of 1% gentian violet in distilled water in/

in 98 mls. of distilled water was used (Whitby and Britton, 1963). The gentian violet slightly stains the nuclei of the leucocytes while the glacial acetic acid haemolyses red blood cells.

A 1/20 dilution of heparinised whole blood in diluting fluid was made and thoroughly mixed in a mechanical mixer. Cell counts were made in a Neubauer counting chamber over 5 mm^2.

(iii) Total White Blood Cells and Differential Counts in the Purified Population

Lymphocyte concentration and purity in the purified cell suspension were routinely assessed under phase contrast in a Neubauer counting chamber. At least 100 lymphocytes were counted. Differential counts were also made using Leishman's stain as above. Interestingly, on four purified cell suspensions stained with Leishman's stain, all cells were small lymphocytes. No large lymphocytes were visible in over 100 cells scanned from preparations made from four different animals.

Cell recovery results for four animals are presented in Table 4. All sheep are breeding ewes from the Damline flock at Skedsbush Farm near Gifford. All values were determined using heparinised blood from the same sample tube. The percentage recoveries range from 15% to 29%. These values are low. However, sufficient cells can be recovered for use in mlct. Therefore, the cell separation procedures were felt to be satisfactory.

Table 4

Recovery of Lymphocytes from Sheep Whole Blood

Using Ficoll-Hypaque Specific Gravity 1.064

Animal	Whole Blood			Purified Cell Suspension		Percentage Recovery
	Total White Blood Cell Count/ml.	Percentage Lymphocytes	Total Lymphocytes	Lymphocytes/ml.	Percentage Purity	
1	6.92×10^6	54	3.74×10^6	8.15×10^5	>99	22
2	1.07×10^7	51	5.46×10^6	0.79×10^5	>99	15
3	6.40×10^6	48	3.07×10^6	0.89×10^5	>99	29
4	4.80×10^6	56	2.69×10^6	0.76×10^5	>99	29

2. The Effect of Complement Dose and of Incubation Temperature

The most suitable test will depend upon the sensitivity required and also upon the time and trouble one is prepared to take. I have compared two forms of microlymphocytotoxicity test. Type A used 5 ul. of rabbit serum as a complement source and incubation took place at room temperature (20 $\pm$ 4°C.). These conditions were chosen because they are the same as those employed for microlymphocytotoxicity testing in the human. Type B utilised 1 ul. of rabbit serum at an incubation temperature of 37°C. This test is the one employed previously. I decided to test the effect of both incubation temperature and complement dose at the same time. This would improve the chances of detecting any differences that existed. If differences did exist, they could be investigated further. The comparison of the two types of microlymphocytotoxicity test showed that using 5 ul. at room temperature gave a very much more sensitive test - at least with sheep antisera directed against sheep lymphocytes and with pooled rabbit serum as a complement source (presented below). I then went on to test the effect of temperature and the effect of increasing doses of complement separately. These results are also presented below.

a) A comparison of the two types of cytotoxicity test

Ten sheep were each tested against 120 antisera in two simultaneous tests. Test A utilised 5 ul. of rabbit serum and the 20 plates were incubated at room temperature (20°C.). Test B utilised 1 ul. of rabbit serum and these twenty plates were incubated at 37°C. All other features of the test were common.

Test plates were prepared and treated identically prior to testing. 2 ul. of undiluted sheep sera was dispensed into each well. Cell concentrations were adjusted to $1.5 \pm 0.5 \times 10^6$ cells/ml. The same cell preparation was used for both tests. The same batch of rabbit serum was used as a complement source for both tests. This was added 30 minutes after the cell suspension. No dye was used and assessment of lysis was made by phase contrast. 2 ul. of fixative was added 60 minutes after addition of complement.

From Table 5 it can be readily seen that there is a much higher proportion of positives when 5 ul. of rabbit serum was added and the test incubated at room temperature. Interestingly, there are very few reactions which are positive when 1 ul. of rabbit serum was used but negative in the other test (9/1,188) or (0.5%). This suggests both tests are detecting the same reactions but one is more sensitive than the other. I then went on to look at the effect of complement dose and temperature separately.

b) Effect of Complement Dose

Here, two paternal half-sibs which have given concordant reactions when tested against over 1,000 sera on several different occasions and are thus believed to have identical lymphocyte antigens, were utilised. Two tests were carried out with a six-month interval. In the first test, ten sera (eight positive and two negative) were titrated in doubling dilutions from 2 ul. neat to 1 ul. 1/16. Cell suspensions from the two animals were then tested against the ten different sera in four tests. For each test 5 ul., 4 ul., 3 ul., 2 ul. or 1 ul. of rabbit serum was added. While the conditions of testing/

Table 5

Comparison of Two Cytotoxicity Tests

Test A

Test B	Number of Positive Reactions	Number of Negative Reactions	
Number of Positive Reactions	178	9	187
Number of Negative Reactions	163	838	1,001
	341	847	1,188

12 reactions were unreadable and are not included.

Test A: 2 stage test using 5 ul. rabbit serum at 20°C.
Test B: 2 stage test using 1 ul. rabbit serum at 37°C.

testing varied between the two sets of four tests, within each test all conditions were standardised except the amount of rabbit serum added. Within each test, the same effect was observed - an increase in titre with an increase in complement dose. Thus, for each dose of rabbit serum, the cumulative scores of 64 titrations were taken. The cumulative score is the cumulative sum of the positive reactions (scored 4, 3, 2, 1, 0) at each of the doubling dilutions tested in each end-point determination. These results are given in Table 6.

These tests were repeated using a different batch of complement. In this second series of tests, ten sera were titrated from 1 ul. neat to 1 ul. 1/32. As before, eight different tests were carried out simultaneously. While each test differed, within each test all treatments were identical except the complement dose. Eighty end-point determinations were made and the cumulative scores compared. Once again within each test a consistent pattern emerged, an increase in the dose of rabbit serum gave an increase in titre. These results are summarised in Table 7. In subsequent tests, 4 ul. of rabbit serum was added to all wells as a complement source. 4 ul. was chosen as a convenient volume to be added with the equipment available and also because there is some suggestion for a plateau at 4 ul.

The reasons for the increase in titre are now discussed. I am only concerned with those variables which will alter with an increase in the dose of rabbit serum. Other parameters (e.g. efficiency of antigen-antibody binding) will be important in determining test sensitivity, but as they do not affect the results obtained here, they have not been considered further.

Table 6

Cumulative Scores of 64 End-point Determinations

ul. of Rabbit Serum used				
5	4	3	2	1
839	842	789	764	724

The cumulative score is obtained by adding all scores (4, 3, 2, 1 or 0) recorded for each dilution of a titration. Cumulative scores have been summarised for 64 titrations at each dose of rabbit serum tested.

Table 7

Cumulative Scores of 80 End-point Determinations

ul. of Rabbit Serum used				
5	4	3	2	1
1250	1205	1133	920	666

The cumulative scores have been summarised over 80 titrations for each dose of rabbit serum.

The increased test sensitivity could have one of three explanations:-

i) Sheep lymphocytes in an excess of rabbit serum could show an increased susceptibility to lysis. Rabbit serum could be a poor medium for keeping sheep lymphocytes viable. This might result in non-specific death or an increased vulnerability to antibody mediated cytotoxicity. This possibility cannot be excluded. However, it seems unlikely as there was no increase in background cytotoxicity in control preparations.

ii) An increase in the levels of active complement might be responsible for the increased test sensitivity. The level of complement in 1 ul. rabbit serum is unlikely to be generally inadequate as 1 ul. of rabbit serum can be diluted out to 1/4 and 100% cytotoxicity still occurs in certain preparations. Several authors have argued that complement is needed in excess because of anti-complementary activity in certain sera (Ferrone et al. 1967; Tiilikainen, 1967) or in certain cell preparations due to e.g. platelet contamination (Svejgaard, 1969; Harris et al., 1970; Smith and Walford, 1970). While the anti-complementary activity of certain sera or cell preparations is not in doubt, this is unlikely to account for the general increase in titre observed with sixteen different sera against lymphocyte preparations from two different sheep.

In any chemical pathway only one stage can be rate-limiting (Dewar, 1965). In antibody-mediated complement-dependent cytotoxicity, the rate-limiting step is unlikely to lie within the complement cascade. Titration experiments usually show/

show that an increased antibody concentration results in increased test sensitivity. Only the concentration of the substrates and enzyme, directly involved in the rate-limiting step, will affect the overall rate in a pathway. As the antibody concentration affects the reaction rate, antibody must be involved in the rate-limiting step. Therefore the rate-limiting step is unlikely to lie within the complement cascade. However, the rate-limiting process could be the activation of complement by bound antibody. This possibility could be tested by specifically increasing the dose of the first complement components of the classical and/or alternative pathways.

iii) The final explanation is that rabbit serum contains natural sub-lytic antibody to sheep lymphocyte antigens. Cross-absorptions with heat inactivated rabbit serum revealed the presence of two anti-erythrocyte antibodies (Chapter 5). One antibody was apparently anti-R. R antigen is known to be present on sheep lymphocytes (Schmid and Cwik, 1975). The other antibody was probably species specific as it was found in 20/20 sheep from diverse origins. The presence of this antigen on lymphocytes is not known.

Further support for the possibility that rabbit antisera contains natural antibodies comes from human cytotoxicity testing. Ferrone (1977) has argued that the superior effectiveness of rabbit complement is due to natural antibody to human lymphocyte membrane antigens. He cites three observations in evidence.

1) There is no relationship between the lytic efficiency of rabbit complement in the lymphocytotoxicity test and the amount of CH_{50} units as measured in the classical haemolytic system. However, the lytic efficiency is related to the level of natural antibody to human lymphocyte antigens as detected by rabbit sera lytic titres towards cultured human lymphoid cells (Ferrone and Pellegrino, 1973).

2) Absorption of natural anti-human lymphocyte antibodies from rabbit serum reduces its lytic capability in the lymphocytotoxic reaction (Ferrone et al., 1971).

3) Addition of sub-lytic amounts of anti-human lymphocyte antibodies to rabbit complement increases the sensitivity of the lymphocytotoxic test (Miggiano et al., 1970; Nelken et al., 1970; Ting et al., 1973).

Arguing by analogy is not always justified but the presence of anti-sheep lymphocyte antibodies in our pooled rabbit sera would provide a reasonable explanation for the experiments reported in this section.

It is difficult to standardise for the presence of these contaminating sub-lytic antibodies mainly because they are sub-lytic. However, it is necessary to be aware of their possible existence and of the variability they could introduce into cytotoxicity testing.

c) Effect of Incubation Temperature

Two experiments were carried out. The first was to decide the effect of temperature in the pre-complement incubation. The second was to decide the effect of incubation temperature in the post-complement incubation phase. In experiment one, two animals, believed to be identical to detectable lymphocyte antigens, were each tested ten times against eight positive and two negative sera. These sera were serially diluted from 2 ul. neat to 1 ul. 1/16. Five of the tests were carried out at a constant incubation temperature of 37°C., the other five were incubated at 20°C. before the addition of complement and at 37°C. subsequently. Within a temperature group, the five tests differed from each other but each test was carried out in an identical manner with a test in the other temperature group except for incubation temperature.

As in the complement comparisons, a cumulative score was given for each of the eight positive sera tested against the two animals in the five different tests at one temperature. The 80 cumulative scores obtained for each temperature were then compared. The significance was calculated by X^2. Results are given in Table 8.

In the second experiment, the same two animals were tested against a different eight positive and two negative sera diluted as above. Once again five tests were made at each of two incubation regimes. While the five tests differed within each incubation regime, each test was carried out in an identical fashion with a test in the other temperature group except for incubation temperature. Both sets of plates were incubated at 20°C. for 30 minutes prior to complement addition.

Table 8

The Effect of Temperature in the Pre-Complement Incubation Phase

Temperature °C.		Cumulative Score	X_1^2	Probability
Pre-Complement	Post Complement			
A 37	37	841		
B 20	37	909	8.46	P <0.01

The cumulative scores have been summarised from 80 individual titrations.

uently, one set was incubated at 37°C., the other at 20°C.

Results were then calculated as above (Table 9). It can be seen that the incubation of the post-complement incubation phase has no apparent effect on serum titre.

The traditional explanation given for the superior effect of 20°C. incubation when compared with 37°C. incubation invokes inactivation of a complement component, probably C3 (D. Bernoco, 1978 - personal communication). A C3 inactivator operates more efficiently at 37°C. and incubation at this temperature results in reduced effectiveness of the complement cascade. The results above with sheep antisera directed against sheep lymphocytes in a cytotoxicity test utilising rabbit complement are consistent with this hypothesis.

3. Choice of Incubation Time

Apart from increasing the dose of rabbit serum, another way to increase the sensitivity of a cytotoxicity test is to increase the incubation time.

After deciding that 4 ul. was the preferred dose of rabbit serum (see previous section on complement and incubation time) and incubation at room temperature was preferable to incubation at 37°C., I resolved to test for the optimum incubation time. Two animals were tested against twenty sera. Seventeen sera were positive and three were negative for these two animals. Ten sera (eight positive and two negative) were alloimmune cattle sera raised by skin grafting. Ten sera (nine positive and one negative) were raised in sheep. Three positive/

Table 9

The Effect of Temperature on the Post-Complement Incubation Phase

Test	Temperature °C.		Cumulative Score
	Pre-Complement	Post Complement	
A	20	37	1,100
B	20	20	1,108

As in Table 8, cumulative scores are a summary of 80 individual titrations.

positive sera were raised by lymphocyte alloimmunisation, the remaining seven were from parous ewes. Each serum was serially diluted out in six doubling dilutions from neat to 1/32. The titre for each serum when tested against each of the two animals was determined for each of ten incubation times. All tests involving the same animal used aliquots from the same cell suspension. All tests were carried out simultaneously and were identical in all aspects except different incubation time.

Interestingly, even after 30 minutes pre-complement and 180 minutes post-complement incubation, there was still no non-specific background killing in the controls. Possible prozone effects were observed in three instances. In two cases after 45 minutes post-complement incubation and once after 60 minutes post-complement incubation. Of the seventeen positive sera, two gave complete positives at the last dilution tested, even at the longest incubation periods. Therefore the end-point could not be determined. These two sera were not included in preparing a summary of cumulative scores. The results of increased incubation on serum titres are presented in Table 10. It is apparent that titres increase with an increase in incubation time. This effect was also consistently observed in all titrations carried out at each of ten different incubation times.

In the summarised results there is a slight plateau between 120 and 150 minutes of post-complement incubation. I believe that this result is not significant. It was not observed in the component sub-totals.

Table 10

The Effect of Increased Incubation Time on Serum Titre

First Batch of Complement

Incubation Time (minutes)		Cumulative Score (Summary)
Pre-Complement	Post-Complement	
30	45	185
30	60	261
30	75	319
30	90	348
30	105	343
30	120	379
30	135	380
30	150	382
30	165	403
30	180	409

The cumulative scores have been summarised for two animals, each tested against 15 positive sera.

When working with farm animals, samples are not usually available until midday. Samples were routinely prepared for testing on the same day. Consequently, it was not feasible to allow a lengthy interval for incubation.

On the basis of the results in Table 10, I decided to allow, in addition to 30 minutes pre-complement incubation, 120 minutes before adding fixative to stop the reaction.

Two batches of complement were used in the type II mlct. I also tested the effect of varying the incubation time with the second batch.

The first set of results is presented in Table 11. This summarises a series of experiments designed to look at the effect of increased dose in the second batch of rabbit serum with increases in incubation time on serum titres. Two cells were each tested, a) at five doses of rabbit serum, b) at four different incubation times and c) against ten positive sera. Two sera were alloimmune cattle sera raised by skin grafting, three sera were alloimmune sheep sera raised by lymphocyte injections and five sera were from parous ewes. Each serum was tested at six doubling dilutions. All tests were carried out simultaneously and treated in an identical manner in respect of all variables and parameters except those mentioned. Each comparison made was identical for all parameters except incubation time.

Here again, there is an increase in test sensitivity, as shown by an increase in titre, with an increase in incubation time.

Table 11

The Effect of Increased Incubation Time on Serum Titre

Second Batch of Complement

Incubation Time (minutes)		Cumulative Score (Summary)
Pre-Complement	Post-Complement	
30	90	1,225
30	120	1,374
30	150	1,393
30	180	1,485

Each cumulative score is summarised for 2 cells at five doses of rabbit serum against 10 positive sera.

Further support for the assumption that an increase in test sensitivity can result from an increase in incubation time is supplied by a second series of experiments carried out with different batches of complement.

Two batches of complement were compared, Batch A and Batch B. Batch A was used for a 120 minute post-complement incubation period. Before using Batch B, I tested this batch at different incubation periods. This was to ensure that the sensitivity of tests using both batches would be the same.

Lymphocytes from four sheep were each tested against ten positive sera. To ensure that each serum sample was positive, different sera were used, where necessary, in each batch of ten. Each serum was tested at six doubling dilutions. Each serum was tested against each cell with two batches of complement. Batch A was allowed an incubation time of 30 + 120 minutes. Batch B was allowed nine incubation times. These ranged from 30 + 60 minutes to 30 + 140 minutes. Increments were of 10 minutes. Allowance was made for the time to add solutions. As before, all tests were carried out simultaneously. All tests were treated identically except in respect of incubation time. Results are summarised in Table 12 and figure 2.

From Table 12 it can readily be seen that 70 minutes post-complement incubation time with complement Batch C gives a test with approximately the same sensitivity as a test with 120 minutes post-complement incubation time with rabbit serum Batch B. All type II microlymphocytotoxicity tests with rabbit serum Batch B had a post-complement incubation time of 120 minutes. All tests with rabbit serum Batch A had a post-complement incubation time of 70 minutes.

Table 12

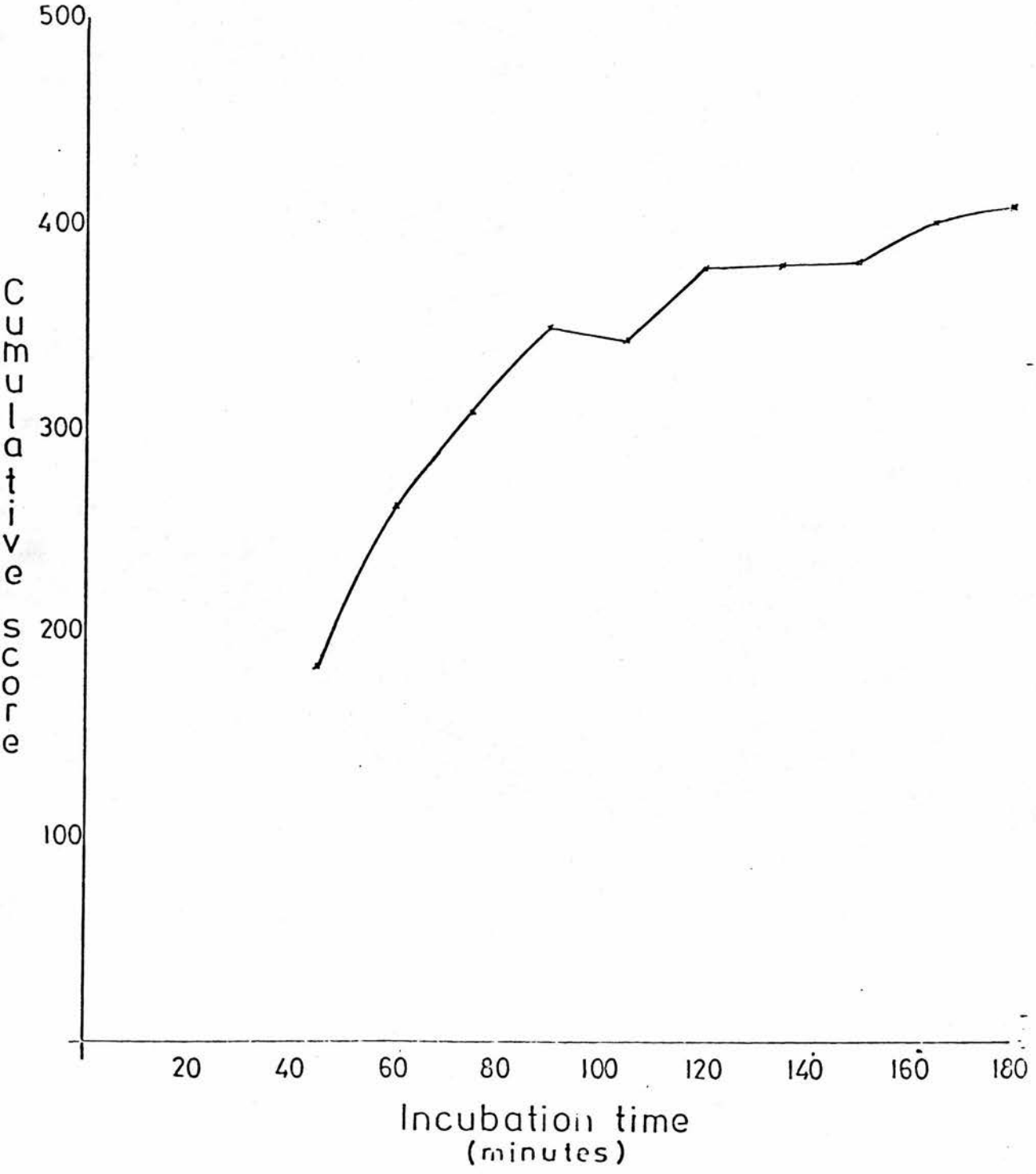
A Comparison of Two Batches of Rabbit Serum as a Complement Source

Batch of Rabbit Serum	Incubation Time		Cumulative Score
	Pre-Complement	Post-Complement	
A	30	120	377
B	30	60	277
B	30	70	343
B	30	80	406
B	30	90	441
B	30	100	470
B	30	110	504
B	30	120	539
B	30	130	565
B	30	140	571

Each cumulative score is a summary of 40 end-point determinations.

Figure 2

Graph showing the effect of incubation time on titre as expressed by cumulative score



In summary, increased incubation time leads to increased test sensitivity. A similar result has been found in the human (Ting et al., 1973). The increase in sensitivity is possibly due to an increase in the number of antigen-antibody reactions or to an increased activation of complement. It could be due to an increased susceptibility to lysis of the cell suspension but this is less likely. In the negative samples, there was essentially no background cytotoxicity even after a total of $3\frac{1}{2}$ hours incubation.

4. Carry-over Effects

One possible source of error in microlymphocytotoxicity testing is carrying-over solutions from one well to another. For example, if a serum has a titre of 1/10, carrying over even 0.1 ul. could result in a false positive reaction. The results of the reproducibility experiments suggested that carry-over effects did not appear to be significant in a type I mlct. However, there was the possibility that carry-over effects could become apparent in a more sensitive test.

Ten cells were screened against fifteen sera at two dilutions on four occasions. Routinely, all suspensions are added by multi-dispenser in the same direction. In this plate layout, every second well contained non-reactive serum. All four replicates were set up simultaneously and treated identically except for plating out solutions.

The first replicate was treated routinely. In the second replicate, after each plating out, the needle tips were wiped. Every second drop was discarded in the third replicate. In the fourth and final replicate, needle tips were wiped then every second drop was discarded. The results are summarised in Table 13.

Only one carry-over was observed in sixty positive reactions. If carry-overs follow a binomial distribution, the 95% confidence limits are from 0-10% (Snedecor and Cochran, 1967). Carry-overs can only occur when the adjacent well is positive. In routine typing of animals, no more than 20% of reactions are positive as a maximum upper limit. Therefore, the upper limit of carry-over effects is 10% of 20% which is 2%. Of this 2%, 20% of these would be expected to carry-over into a positive well, therefore only 1.6% would be of any effect. As this 1.6% is the maximum possible effect due to y-over, it seemed reasonable not to change routine procedures.

Other arguments can also be advanced in favour of not changing existing procedures. In this test above, the sera were not titrated. Some of the undiluted sera would be stronger than those used for routine testing. Stronger sera would be more likely to produce carry-over effects. Additionally, both alternatives to reducing possible carry-over effects, wiping and discarding, are tedious and time-consuming. Discarding every second drop also wastes reagents. With wiping, there is the possibility of drawing fluid from the tips of the needles into the tissue. This would cause the dispensers to dispense less than the required volumes.

Table 13

Incidence of Carry-over Effects in a Type II Mlct.

Replicate	Treatment	Total Tests	Total Positives	Carry-overs
1	Normal	300	60	1
2	Wipe	300	60	0
3	Discard	300	60	0
4	Wipe and Discard	300	60	0

In summary, this section has shown that routine procedures can produce a small carry-over effect. This effect is, however, at maximum, unlikely to produce many errors. These errors can be eliminated but the procedures for doing so are unsatisfactory and likely to introduce other errors which cannot be so readily noticed. In the light of this, it was decided to continue using routine measures but to take note of this as a possible source of error.

Summary

This section has described the consequences of changing several of the parameters of the microlymphocytotoxicity test. In order to increase the sensitivity of the test, several parameters were altered to form a type II mlct. The type II mlct. is now described.

5. Type II Microlymphocytotoxicity Test

Materials and Methods

Materials

Ficoll-Hypaque: A 12% Ficoll solution was added to an equal volume of a 15.5% sodium diatrizoate solution. When necessary, the specific gravity was adjusted with either of the two solutions to give a final specific gravity of 1.064 ± 0.001.

Washing Solution: This was unchanged as Hank's Balanced Salt Solution with 10% pooled non-reactive ram's serum. No bicarbonate was added. The pH of this solution was always in the range pH 7.3 ± 0.1.

Complement: Two batches of unabsorbed pooled rabbit serum were used as a source of complement. These batches were stored as before.

Eosin: A 5% aqueous stock solution was stored frozen at -25°C. Aliquots were thawed when necessary and kept in the dark at 4°C. Immediately prior to use, equal volumes of the 5% stock solution and 2 X HBSS + 20% NRSS were mixed to give a final concentration of 2.5% eosin in HBSS + 10% NRSS.

Fixative: 40 mls. of 40% formaldehyde solution (formalin), was made up to 100 mls. with 55 mls. of 0.9% saline and 5 mls. 0.15M Na_2HPO_4.

Terasaki Trays: These were prepared as before using 1 ul. or 2 ul. neat or diluted sera in each well.

Methods

Blood Collection: This was carried out as before.

Lymphocyte Separation: 2 mls. of heparinised whole blood was carefully layered onto 2.5 mls. Ficoll-Hypaque. This was then centrifuged at 1,500 g. for 20 minutes. The lymphocyte rich interface was removed and washed twice in HBSS + 10% NRSS. Each wash was at 100 g. for 15 minutes. Cell pellets were resuspended in 0.5 mls. HBSS + 10% NRSS and assessed for viability, purity and lymphocyte concentration under phase contrast. Cell concentrations were then adjusted to 1.5×10^6 lymphocytes/ml. by adding HBSS + 10% NRSS. Erythrocyte contamination was always less than 5%. Granulocyte contamination was usually negligible but always less than 2%. Platelets were not seen.

Cytotoxicity Testing: 1 ul. of cell suspension was added to each well. After 30 minutes at 20 ± 4°C., 4 ul. of rabbit serum was added to each well. 1 ul. eosin suspension was added after 50 or 100 minutes depending upon the complement batch. After a further 20 minutes, fixative was added to fix the reaction. Plates were read and scored as before.

6. Reproducibility

A specific test to determine reproducibility was not carried out. It was felt that specifically testing for reproducibility might bias the results and lead to artificially high values.

However, in the course of testing, a small number of animals were tested against the same sera on more than one occasion. Reproducibility values were calculated from the repeat tests. Tests were scored as positive or negative only. Repeat tests were of two types. Firstly, animals were tested against the same layout of sera. Animals were tested against sera in groups. A number of test-trays were prepared, each having an identical serum layout. Occasionally, the same animal was tested more than once against the same test-tray. 1,474 repeat tests were carried out. There were 79 discrepancies. The estimate of reproducibility is 94.6%.

The second estimate of reproducibility comes from repeat tests performed on different plate layouts. 2,375 repeat tests were carried out. There were 106 discrepant results. The estimate for reproducibility is 95.5%. This estimate is more reliable than the first/

first estimate. Many of the repeat tests, using the same plate layout, were made at the same time as the original tests. However, the two estimates are very similar. The similarity suggests that the plate layout has very little effect on the reproducibility.

The overall levels of reproducibility are slightly lower than values reported in the human using a similar test (Perdue et al.,1977). However, the reproducibility is quite acceptable for microlymphocytotoxicity tests. However, within certain values, the level of reproducibility is perhaps not as important as the fact that the reproducibility is known. When clustering sera and assigning antigens, it is important to know the reproducibility in order to allow for false positive and false negative reactions. This point is considered in more detail in Chapter 5.

Discussion

A test to determine antibodies directed against lymphocytes should be sensitive, reproducible and convenient for testing large numbers of animals and sera. The tests described in this chapter appear to fulfil the three criteria. Each criterion is discussed separately.

The sensitivity of a cytotoxic test depends upon many parameters (e.g. avidity of antibody, efficiency of complement activation and antigen density). Many parameters will not vary between tests. They have not been considered further. Several variables which might be expected to alter test sensitivity were investigated. They were target cell vulnerability, level of complement, incubation temperature and incubation time.

Different cell types are more or less vulnerable to antibody-mediated lysis. Also, the vulnerability of the same cell type to antibody-mediated lysis varies with environmental conditions. Dying cells might be expected to be lysed more readily than healthy cells. Therefore greater test sensitivity could be expected from the use of a more vulnerable cell suspension. However, a background of cell death makes viability more difficult to assess. This could lead to an increase in reading errors on the test error rate. The important point is that vulnerability should be consistent between tests. The levels of background cytotoxicity in all tests never rose above 5%. This suggests that vulnerability of cell suspensions did not differ significantly with different tests.

The sensitivity of the microlymphocytotoxicity test was shown to be increased by -

i) increasing the volume of rabbit serum in the test,
ii) changing the incubation temperature from 37°C. to 20°C.
iii) increasing the incubation time.

Possible explanations for these phenomena have already been discussed.

Greater increases in sensitivity could perhaps have been achieved by even higher doses of rabbit serum, even longer incubation times or by larger volumes of antisera. However, the procedure followed was convenient, reliable and sufficiently sensitive for practical purposes.

The results obtained in lymphocytotoxicity tests also need to be reproducible. The three major sources of error in lymphocytotoxicity tests are random, sera related and lymphocyte related. Random errors are caused by mishandling and reading errors. These are believed to form the largest source of error in the tests and fortunately are the easiest to minimise. By entering each result directly into a table and not via protocol sheets, I hope to have reduced the occurrence of reading errors. When 6,000 wells were re-read in a blind trial, only three wells were scored differently. Errors in dispensing can be of two types, operator related or equipment related. By adding all solutions by multi-dispenser, operator related errors due to adding too little or too much of any of the components will be present in all the wells of a column and more readily noticed. Trials with every second column as a duplicate column, showed that this was not a problem.

Sera related errors are due to carry-over effects, working at the end-point of a positive serum and the use of poorly supportive sera. Carry-over effects are caused by the tip of the needle carrying a small amount of a positive serum into a negative serum well (Mittal et al., 1968). Carry-over effects did not appear to be a major problem in my tests. Working at the end-point of a sera is a familiar problem and a major source of irreproducibility between tests. Due to small variations between tests which cannot easily be controlled, the highest dilution (titre) giving 100% lysis in tests can vary quite considerably. If a low titre sera is used or a high titre serum used near its end-point, lysis of lymphocytes from the same animal will vary between tests. Sera used for typing/

typing were titrated and used at two dilutions above the end-point to minimise this problem. "Poorly supportive" sera are those which appear to cause a low degree of non-specific cell death. While this does not appear to be a problem with sheep or rabbit sera, it is noticeable that sheep cells when tested against cattle sera show a low degree (<10%) of background lysis. This may be due to cattle complement or even lytic enzymes present in the media. If lysis is due to these effects, it can be avoided by heat inactivating the sera before use. All sera used in typing were sheep sera. All sera were heat-inactivated at 56°C. for 30 minutes.

The third source of error in tests is lymphocyte related. These errors can be caused by using contaminated, poorly viable or "pathological" lymphocytes.

Contaminated lymphocyte preparations cause reading difficulties due to the similar appearance of granulocytes and lymphocytes. Also especially in the case of platelets, contaminating cells may react with the antibodies of the antisera and affect all cell lysis. Poorly viable cells may die in the course of the incubation reaction and thus mimic antibody specific cell lysis. Thus, causing a negative reaction to appear positive. Only suspensions with a viability of >95% were used. In certain human diseases (e.g. chronic lymphocytic leukaemia) the proportion of B lymphocytes to T lymphocytes in peripheral blood changes markedly. Lymphocytes prepared from patients with chronic lymphocytic leukaemia are nearly all B lymphocytes.

The reaction patterns of the B lymphocytes with typing reagents are different from those observed with normal cells. The differences are probably due to the typing reagents being contaminated with specific antibodies against B lymphocyte antigens (e.g. Ia antigens). As the proportion of B to T lymphocytes in peripheral blood is usually quite small, the contaminating antibodies to B lymphocyte antigens are not usually noticed. In order to avoid this problem, only apparently healthy sheep were used to provide lymphocyte samples.

The final requirement of a lymphocytotoxicity test is that it is suitable for large scale testing. The test used here is a modification of the micro test introduced by Terasaki and McClelland (1964). This test is very conservative of reagents and very quick. Altering the interval between adding eosin and adding fixative to twenty minutes allowed a larger number of tests to be carried out more easily. With this modification, up to 4,800 tests (80 plates) could be carried out on one day.

In summary, the cytotoxicity test described here would appear to be suitable for screening antisera and also for detecting lymphocyte antigens. The results of screening antisera are described in Chapter 4. The detection of lymphocyte antigens is described in Chapter 5.

CHAPTER 4

THE PRODUCTION AND ACQUISITION OF ANTISERA

Introduction

A. Naturally Occurring Antibodies

Introduction

Materials and Methods

Results

1. Presence of Cytotoxic Antilymphocyte Antibodies in Sheep.
2. Parity and the Incidence of Sheep with Cytotoxic Antibody.
3. The Time of Development of Anti-Foetal Antibody.
4. The Persistence of Antibody Activity.

Discussion

B. Antisera Produced by Deliberate Immunisation

Introduction

Materials and Methods

Results

1. Lymphocyte Immunisations
2. Skin Grafting

C. Foreign Sera

D. Selection of Sera for Use as Typing Reagents

Introduction

The purpose of this chapter is to describe how antisera were produced and collected. Historically, allogeneic differences in cell surface antigens have been detected by antisera from three distinct sources. These are naturally occurring antibodies, antisera produced by deliberate immunisation and finally antibodies produced in cell culture, monoclonal antibodies. The production of monoclonal antibodies was first carried out by Kohler and Milstein (1975). Since then the technique has attracted considerable interest and is likely to prove of tremendous potential in the future (Williams, 1979).
However the technique still demands considerable expertise. I did not use this method to raise antisera for this project. Use was made of both naturally occurring antibodies and of antisera raised by deliberate immunisation. This chapter consists of four sections - naturally occurring antibodies, antisera produced by deliberate immunisation, foreign sera and selection of sera for use as typing reagents. Each section is reviewed and discussed separately.

A. Naturally occurring Antibodies

These antibodies are of two types. Firstly, natural antibodies may be produced in response to a ubiquitous antigenic stimulus such as food or microorganisms. Potentially all immunocompetent members of a species, not themselves possessing the antigen, could respond to these antigens. Consequently such antibodies occur randomly in immunocomponent individuals. It is believed that antibodies to the A and B blood groups in man, anti-J in cattle and anti-R in sheep are of this type. As far as I am aware, anti-leucocyte antibodies of this type have seldom been found in any species so far studied.

Ebringer (1978, 1979) has suggested that gram negative bacteria have antigens that cross-react with antigens in lymphocyte membranes. More specifically he quoted results obtained in Australia, that antibodies raised against HLA-B27 react with three gram negative organisms Enterobacter aerogenes, Klebsiella pneumoniae and Yersinia enterocolotica. If this work is confirmed then one would expect to find anti-HLA-B27 in serum from a proportion of normal donors. Additionally, if other micro-organisms carry antigens similar to other lymphocyte antigens, antisera to other lymphocyte antigens should also occur. The fact that naturally occurring antisera have not so far been commonly reported would argue against Ebringer's interpretation. Although Collins et al. (1973) and Lepage et al. (1976) have both found naturally occurring antibodies in human sera, Ebringer's hypothesis could provide an explanation for their results.

The second type of naturally-occurring antibodies directed against allogeneic tissue antigens are those found only in sera from parous females.

Anti-leucocyte antibodies have been found in humans (Paynes and Rolfs, 1958, van Rood et al., 1959), rhesus monkeys (Balner et al., 1965c), sheep (Ford and Elves, 1974), goats (van Dam et al., 1976), pigs (White, 1974) and cattle (Newman and Hines, 1979). Anti H-2 haemagglutinins have also been detected in parous mice (Herzenberg and Gonzales, 1962; Goodlin and Herzenberg, 1964; Kaliss and Darg, 1964). In humans (Payne, 1962; Terasaki et al., 1970), sheep (Ford and Elves, 1974) and mice (Kaliss and Darg, 1964), naturally occurring antibodies have been/

been looked for and not found in males and nulliparous females. This implies that pregnancy is a necessary prerequisite for the production of antibodies. In the other species this result is inferred. Stone (1978 - personal communication), has detected cytotoxic antibodies in the sera of young calves. These were presumably absorbed from the colostrum.

Early reports (Payne, 1962; Terasaki et al., 1970) concluded that two or more pregnancies were usually required for the production of anti-leucocyte antibodies in humans, although Overweg and Engelfriet (1969) found a single pregnancy was sufficient. Subsequent work by J. G. Bodmer (1973) has shown that a single pregnancy is an adequate stimulus for the production of cytotoxic antibodies. She showed that 20-27% of primiparous women tested at 32 weeks of gestation produced detectable anti-HLA antibody. The differences are probably due, at least in part, to the use of more sensitive techniques.

In this study, it was not possible to obtain a flock of sheep for my exclusive use. Consequently, sheep were used simultaneously in several experiments. It was not often feasible to carry out immunisation procedures on sheep as this would interfere with other experiments. Therefore the suitability of parous sera as typing reagents was investigated. In humans, parous sera provide the main source of typing reagents.

In a previous study, C. H. J. Ford used parous sera as typing reagents in sheep (Ford, 1974). However he did not show mendelian segregation in families and provided no evidence of simple genetic control of lymphocyte antigens (Ford, 1975). This may have been a consequence of the low titre of his sera, Ford (1973) states that their titres were usually 1/4 or less.

Before attempting to use parous sera as typing reagents, further information was sought about the occurrence, persistence and time of production in pregnancy of lymphocytotoxic sera in sheep. As it was seldom possible to use the fathers or the offspring to check serum cytotoxicity, a heterogeneous unrelated panel of sheep from several breeds was used for screening purposes. Additionally, as pointed out by Ford (1973), complete obstetric records for sheep are rare. Information on abortions and stillbirths are not always available. Also, multiple births are more common in sheep than in humans. This tendency is especially marked in certain breeds. For ease of presentation, each birth event is referred to as a lambing, irrespective of how many births occurred at the lambing.

Materials and Methods

Serum Collection

Whole blood was taken by jugular venepuncture into 7 ml. dry, evacuated siliconed glass vacutainer tubes (Becton-Dickinson, England). In certain cases animals were resampled and large volumes of blood taken (200-400 mls.). Large volumes were taken by jugular venepuncture into dry evacuated glass bottles. All samples were left at room temperature for 24 hours to allow clots to retract. Clots were discarded, the sera were centrifuged at 1,500 g. for 25 minutes to remove cells, platelets and debris, and subsequently stored at -20°C. On thawing all sera were heat-inactivated at 56°C. for 30 minutes.

Animals

A diverse collection of lambs, ewes and rams from several breeds were sampled. All animals were over 3 months of age. Sheep were drawn from the four Scottish farms of the Animal Breeding Research Organisation.

Cytotoxicity Testing

Lymphocyte samples were prepared and tested against undiluted and 1/8 dilutions of sera in a type I mlct. Details of this method have been provided in Chapter 3.

Repeat Bleedings

Repeat bleeds were taken from animals with positive sera whenever possible. The intervals between bleedings varied according to other commitments and husbandry arrangements.

Results

1. The Presence of Cytotoxic Anti-lymphocyte Antibodies in Parous Sheep

Serum samples were drawn from 530 sheep. No animal had previously been deliberately immunised against allogeneic tissue. Each serum was tested blind in a type I mlct. against lymphocytes prepared from 20 to 60 different sheep. All parous ewe samples were tested against cells from, at least, 41 different sheep. In total, lymphocytes from over 240 sheep from 11 different breeds or cross-bred flocks were utilised for screening purposes. Serum samples causing an increased cell death of at least 40% relative to the background in one or more animals/

animals, were deemed to contain cytotoxic anti-lymphocyte antibodies. The occurrence of this type of antibody in parous sheep, non-parous sheep and rams is shown in Table 1.

The results in Table 1 show that naturally occurring cytotoxic anti-lymphocyte antibodies can only be detected in parous sheep. This suggests that the antigenic stimulus is provided during pregnancy. This stimulus can conceivably come from only the mother or the foetus. Pregnancy could cause the ewe to produce anti-self antibodies. These could be raised against maternal tissue or genetically controlled foetal antigens inherited from the mother. In the course of screening parous sera, 47 positive sera were tested against the serum donor. All 47 sera were non-reactive with the donor. This suggests that autoantibodies are not responsible for the observed cytotoxicity of parous sera. They may occur, but if so, it must be at a low frequency.

The only other source of immunogenic material is the foetus. Here antibodies could be produced against the paternally inherited antigens of the lambs. Alternatively, antibodies might be produced against foetal antigens not present in either parent. Foetal antigens in this latter category include foetal stage-specific antigens and possibly foetal antigens which are not under genetic control. To discriminate between these latter two hypotheses, serum samples were collected from 26 primiparous ewes four weeks post-partum. A further 8 ewes were bled in the interval, 2 weeks before to 2 weeks after lambing. These samples were all tested against the relevant ram and also against a heterogeneous panel of

Table 1

The Occurence of Antilymphocyte Antibodies in Sheep

Sheep Category	Number of Animals without Antibody	Number of Animals with Antibody
Parous Ewe	233	164
Non-Parous Ewe	90	0
Ram	43	0

41 largely unrelated sheep. Only four of the 34 samples contained detectable cytotoxic anti-lymphocytic antibody. These four samples were all positive against one or more of a panel of 41 sheep and also against the relevant ram. The other thirty samples which were negative against the panel were also negative against the ram. This implies that these antibodies are probably directed against the paternal antigens of the father.

2. Parity and the Incidence of Sheep with Cytotoxic Antibody

Serum samples came from a variety of flocks. They were quite heterogeneous as to size, inbreeding coefficients, breed and husbandry arrangements. Also, the interval between the last lambing and the time of sample collection varied quite considerably between animals. For these reasons, it did not seem sensible to pool data from different sources.

In sheep husbandry, all ewes in a flock are usually mated ('tupped') during a restricted period of time. The lambing dates of the fertile matings usually occur within a six week period. Mating is usually annual but certain breeds and cross-breeds can produce two crops of lambs a year. Therefore samples drawn at the same time from the same flock will minimise the effect of different time intervals between lambing dates and sampling times.

There were three instances where a reasonably large number of sheep from an outbred flock were sampled on the same occasion. These provide some information on the association between parity and the incidence of cytotoxic antibody for these flocks. These three examples are presented separately below.

(i) Thirty-eight primiparous ewe-lambs of the Dam Line breed at Skedbush farm were bled four weeks post-partum. Serum samples were tested against forty heterogeneous and largely unrelated sheep. Six of these thirty-eight samples were positive. These results are presented in Table 2.

(ii) One hundred and one ewes of a cross-bred flock (Finnish Landrace x Dorset Horn) were bled six weeks after lambing. To ensure bleeding to the nearest week, bleeding was spread over six weeks. In this flock, unlike most, a proportion of ewes can lamb twice in a year. The sheep were bled after the third lambing session. Due to a proportion of barren matings and aborted pregnancies, not all sheep managed to lamb at every opportunity. All ewes produced offspring at the previous lambing. The results are presented in Table 3.

(iii) In this trial, 108 ewes were sampled. They were sampled over three days in July 1977. The fertile sheep would have lambed some 2-3 months earlier. These sheep were five or six years old. All came from the flock of Scottish Blackface sheep maintained at Stanhope Farm. The five year old sheep were mated on four occasions and the six year old sheep on five occasions. As above, due to a proportion of barren matings and aborted pregnancies, not all sheep lambed at every opportunity. Of the 108 samples tested, seventy two were positive and thirty six were negative. The results are broken down in Table 4. Lambing histories for four animals are not known with certainty and they are not included in the summary of results./

Table 2

Incidence of Cytotoxic Antibody in Sheep

Four Weeks after the First Lambing

Number of Lambings	Number of Sheep with Antibody	Number of Sheep without Antibody	Total Sheep Tested	Percentage of sheep with Antibody
1	6	32	38	16

Table 3

Incidence of Cytotoxic Antibody and the Number of Lambings in a Flock of Crossbred Sheep

Number of Lambings	Number of Animals with Antibody	Number of Animals without Antibody	Percentage of Sheep with Antibody
1	9	18	33
2	25	43	37
3	1	0	100

Table 4

Incidence of Cytotoxic Antibody and the Number of Lambings in a Flock of Scottish Blackface Sheep

Age of Ewe	Number of Lambings	Number of Animals with Antibody	Number of Animals without Antibody	Percentage of sheep with Antibody
6 years	2	0	1	0
	3	3	0	100
	4	10	3	77
	5	16	8	67
5 years	1	0	1	0
	2	5	2	71
	3	16	7	70
	4	18	14	56

results. This flock, which consists of older ewes, has a high frequency of sheep carrying cytotoxic antibodies. When broken down, this does not correlate too well with the number of lambings.

Overall the results of these three experiments have shown:-

i) Pregnancy is a necessary prerequisite for the production of cytotoxic anti-lymphocyte antibodies.

ii) Even a single pregnancy can elicit a detectable antibody response.

iii) The proportion of sheep carrying detectable antibodies varied in different flocks. Within flocks, there was no apparent increase with parity in the proportion of sheep carrying cytotoxic anti-lymphocyte antibodies.

3. The Time of Development of Anti-Foetal Antibody

Having established that events in pregnancy are responsible for the production of cytotoxic antibody, I wished to establish when these events took place. If the antibodies were produced at the same stage in pregnancy or post-partum this would allow me to determine the optimum time for bleeding ewes to obtain sera which could be used as typing reagents.

Thirty-four ewe-lambs, not previously mated, were mated to one of four rams. All sheep were Damline animals from Skedsbush farm. The ewes were bled serially throughout pregnancy at approximately three week intervals. Additionally, twenty-six of the thirty-four sheep were also bled four weeks post-partum.

All sera from the pregnant ewes were tested neat and at dilutions of 1/2, 1/4, 1/8 and 1/16. All tests with one exception were performed with freshly prepared ram lymphocytes. The exception was the serum from 7J456 bled on 5.5.78. This was positive at 1/16. Subsequent determination of titre of this serum was made with frozen ram lymphocytes. Only four of the 34 animals produced antibodies.

a) Time of Antibody Production

The time at which antibody was first detected varied between the four positive animals (Table 5). In one animal, antibody could be detected at 55 days; in the other animals, no antibody could be detected until 91, 99 and 111 days. As the gestation length in this breed of sheep is approximately 140 days, the antibody production was apparent by the beginning of the second trimester in one ewe and at the beginning of the third trimester in the other three ewes.

b) Titres after Parturition

Only two of the four positive sheep could be sampled after lambing. One of these showed an increase in titre to 1/32. The other showed a decrease in titre from 1/4 to positive only when undiluted.

In humans, there appears to be no standard time of antibody production. A small proportion of pregnant women produce antibody early in gestation. The proportion of females carrying antibody increases steadily throughout pregnancy. The results obtained here suggest that a similar situation exists in sheep.

Table 5

Titres of Positive Sera in the Four

out of 34 ewes Reacting to a First Pregnancy

Ewe	Tupping Date	Date of Bleed						
		21.12.77	12.1.78	2.2.78	2.3.78	17.3.78	3.4.78	4 weeks Post-Partum
447	18.11.77	-	-	-	N	1/2	1/4	N
012	23.11.77	-	N	1/4	1/4	1/4	1/2	/
450	1.12.77*	-	-	-	1/2	1/4	1/4	/
456	11.11.77	-	-	-	N	N	1/4	1/32

*This date is not known with certainty and is an approximation based on the lambing date and average gestation length.

N = positive at undiluted only

/ = not done

- = no reaction detected

c) Proportion of Responders

A further 12 animals were sampled through pregnancy and at four weeks post-partum. Due to the untimely death of one of the rams involved, the serum samples could not be tested against the sire of the lambs. However, the post-partum samples were tested against the same panel of 41 lymphocytes. Of the twelve, four were positive. This makes a total of eight positives in forty-six tested samples (17%). The sample size is too small to give more than an indication of the incidence of antibody formation.

Possible causes for the loss of titre of the post-partum bleed are discussed later.

4. The Persistence of Antibody Activity

55 parous ewes whose sera gave interesting reactions were re-bled. The interval between sampling varied between 32 and 449 days. The length of the interval depended upon husbandry arrangements and other commitments. The second and any subsequent bleeds were re-tested against an identical or a very similar panel of sheep. Results obtained from a comparison of the serum samples are presented in Table 6.

There are several points of interest in the Table. Firstly, many animals have serum titres which are remarkably persistent over time. 41 sheep showed no marked variation in titre or reaction frequency. This probably reflects a continuing low level of antigen stimulation within the host. Slight differences in titre are probably due to test variation.

Table 6

Persistence of Antibody Activity in Parous Ewes

Serum Donor Identity	Interval Between Sampling (Days)	Titre at First Bleed	Titre at Second Bleed	Difference Between Bleeds
1N065(a)	32	1/32	1/8	˅
1N195	32	1/8	1/8	-
2N197	32	1/8	1/8	-
2N212	32	N	N	-
2N223(a)	32	1/128	1/64	-
1T122(a)	33	1/32	1/16	-
1T393	33	1/16	1/16	-
1T414(a)	33	1/8	1/8	-
2T062	34	N	N	-
2T095	34	1/8	1/8	-
4E40(a)	118	1/8	1/8	-
3E16	118	1/16	1/32	-
1732	118	1/32	1/16	-
1N065(b)	119	1/8	1/16	-
1T122(b)	120	1/32	1/16	-
1T414(b)	120	1/8	1/8	-
5Z8	130	1/4	1/8	-
1N006	152	1/8	1/8	-
1N019	152	1/32	1/8	˅
1N065(c)	152	1/32	1/16	-
1N128	152	1/8	1/8	-
1N174	152	1/8	1/8	-
1T122(c)	153	1/32	1/64	-
1T414(c)	153	1/8	1/8	-
1T387	154	1/8	1/8	-
5Z4	160	1/8	1/8	-
D505	184	1/4	1/4	-
4S8*1	265	1/4	N	˅
BL8*1	274	1/4	N	˅
23095*1	274	N	1/8	^
4G105*3	274	N	N	-
2T098*2	354	1/8	1/4	-
4E40(b)*2	355	1/8	1/16	^
2N045*1	356	1/8	1/8	-
2N065*1	356	N	N	-
2N142*1	356	1/16	1/8	-
2N149*1	356	N	0	-
2N212(b)*1	356	1/16	N	˅
2N216*1	356	1/8	1/8	-
2N223(b)*1	356	1/8	1/128	^
2T031*1	370	1/8	1/8	-
2T075*1	370	1/32	1/8	˅
2T093*1	370	1/8	0	˅
2T095*1	370	1/8	1/32	^
2T098*2	370	1/8	1/8	-
2T110*1	370	1/8	1/4	-
2T154*1	370	1/8	1/8	-
2T156*1	370	1/16	1/8	-
2T238*1	370	1/32	1/8	˅
2T278*1	370	1/64	1/256	^

Table 6 (continued)

Serum Donor Identity	Interval Between Sampling (Days)	Titre at First Bleed	Titre at Second Bleed	Difference Between Bleeds
2T374*M	370	1/4	1/32	^
2T389*1	370	1/32	1/128	^
2T229*1	371	1/16	1/16	-
2T275*1	371	1/16	1/16	-
2T288	449	1/8	1/16	-

Key

*1 These animals had an intervening pregnancy at which one lamb was born.

*2 These animals had an intervening pregnancy at which twin lambs were born.

*3 This animal gave birth to triplets in an intervening pregnancy.

*M This animal had a miscarriage late in pregnancy.

\- No marked change in titre or reaction frequency.

v Decrease in titre and reaction frequency of at least two doubling dilutions.

^ Increase in titre and/or reaction frequency of at least two doubling dilutions.

Secondly, eight sera showed a fall-off in titre. This fall-off appears to be independent of the original titre. Low titre sera are the most likely to fall below the resolution of the test and become negative (e.g. 2N149 and 2T093).

Finally, seven sera showed a marked increase in titre or reaction frequency on the subsequent bleed. These were 23095, 4E40(b), 2N223(b), 2T095, 2T278, 2T374 and 2T389. All these donors had a subsequent pregnancy which would provide the necessary stimulus. Of the twenty-seven serum donors which lambed between bleeds, these seven were the only animals which showed a detectable increase in serum titre or reaction frequency against a similar or identical panel of test cells. This figure (seven out of twenty-seven) is higher than that found for the immunisation of primiparous ewes (29% versus 17%). It is not significantly greater (X^2 1 d.f = 1.56, $0.2<P<0.3$). However, the small sample size and the possibility that not all animals which responded were detected, mean that the comparison is not conclusive.

In summary, this section shows that many sera retain their titres even in the absence of further stimulus. Other sera show a pronounced loss of titre. Within the time course of this experiment, certain low titre sera lost all activity completely. This is in agreement with results obtained in other species and other antibody systems. My results provide no explanation for this.

Discussion

There is a wide literature on the occurrence of anti-lymphocyte antibodies in pregnancy in several species. It was considered valuable to review the material potentially relevant to sheep at this point.

Presence of Cytotoxic Anti-lymphocytotoxic Antibodies in Sheep

Data presented here shows that a high proportion of non-immunised parous sheep contain cytotoxic anti-white blood cell antibodies in their serum. Of 397 parous ewes tested, 233(59%) had detectable antibodies in their serum. This confirms the earlier study by Ford and Elves (1974) who found 52.1% of 119 parous ewes possessed anti-leucocyte antibody. The proportion of positive females is higher than those reported in women. Terasaki & McClelland (1964) reported that 49% (30/61) of women who had five or more pregnancies had detectable cytotoxic anti-leucocyte antibody. Similarly, Naito et al. (1970) examined women with four or more pregnancies and found a frequency of 49% positives (82/167). Women with fewer pregnancies had a lower incidence of antibody. Thus, in Terasaki & McClelland's study (1964) only 16% of women with one to four pregnancies possessed antibody. In a later study, Terasaki et al. (1970) found incidences of 24% and 36% in two and three pregnancy women respectively. While Sever & Terasaki (1970) found that only 16-21% of women who had been pregnant three or more times contained antibody. Ford and Elves (1974) suggested that the higher incidence of antibody in ewes may be a consequence of sample size or alternatively due to the higher incidence of multiple births in ewes than in women. As the result has been/

been repeated on a larger sample, this suggests that it is not an rration due to the statistical effects of small sample size.

Nonetheless, the greater ease of immunisation in sheep may be more apparent than real. Early workers used less sensitive techniques and were often unwilling to assign antibody activity to low reaction frequency and/or low titre sera. Certainly recent studies in the cow have shown a high incidence, comparable with the sheep studies, of cytotoxic anti-white blood cell antibodies in parous cattle (Newman, 1979 - personal communication).

Other factors alone, or in conjunction with each other, may help to explain differences. These could include the parity of the females sampled and also the interval between parturition and the time of sample collection. Additionally, certain populations may have suffered the stochastic effects of random drift and inbreeding. These effects could have led to increased homozygosity in specific populations. Increased homozygosity would reduce the average genetic disparity between mother and offspring, and this could reduce the incidence of cytotoxic antibody formation in pregnancy. The history of certain sheep breeds (Alderson, 1978) and husbandry arrangements in the sheep flocks I studied, suggest that a proportion of sheep breeds and flocks may show increased homozygosity. Some, but not all, studies of biochemical polymorphisms in sheep have shown reduced levels of polymorphism (Brewer and Sing, 1969, McDermid et al., 1975; Baker and Manwell, 1977). The difference may be due to the use of different breeds and flocks in the different studies. In addition, Oh and Maclean (1975), have compared the antigen frequencies in the human population and the antibody specificities produced/

produced as a result of pregnancy. As a result of this, they have suggested that the immunogenicity of different antigens in the major histocompatibility complex varies. Conceivably therefore, the immunogenicity of the antigens in a population and their relative frequency will alter the incidence of females bearing anti-lymphocyte antibodies. In summary, several different effects could alter the incidence of antibody. As these effects are largely uncontrolled in the published studies on the incidence of antibody in sheep and humans, detailed comparisons may not be very significant.

Parity and the Incidence of Antibody Production

I have not pooled all my heterogeneous data on parity and the incidence of antibody production. Instead, I have presented three reasonably homogeneous samples. Within these samples the incidence of cytotoxic antibody does not appear to increase with increasing parity. This evidence however is by no means conclusive. This may be a consequence of the small sample size. Alternatively, the date of the latest lambing varied between sheep sampled. Some of the matings were barren and sheep would not have lambed for up to fourteen months prior to sampling. As results presented earlier showed that some cytotoxic antibodies decay with time, this may have affected the results.

Terasaki et al. (1970) have suggested that in humans the incidence of antibodies does not increase after four pregnancies. Caldwell (1978 - personal communication) has found a similar plateau effect in cattle. My data are too heterogeneous to allow me to determine if/

if a similar situation exists in sheep.

The incidence of primiparous ewes with antibodies was determined as 17% (8/46). Six of the negative animals were not bled later than one week post-partum and the other forty animals were not bled later than four weeks post-partum. Possibly subsequent bleeds and/or more sensitive techniques would have detected a higher incidence of cytotoxic antibody. Overweg and Engelfriet (1969) studied the formation of leucocyte isoantibodies in first pregnancy women. Eleven women developed antibody during the pregnancy, while four developed antibody subsequently in the period, two to four weeks post-partum. If an analogous situation exists in sheep it is possible that some of the six negative animals not bled more than one week post-partum might have subsequently developed antibody.

Ven der Werf (1971) has shown in humans that cytotoxic antibody can be induced at quite an early stage in pregnancy. He reported that one woman had anti-foetal antibody as early as the first trimester of the first pregnancy. Similarly in sheep, the presence of antibody was confirmed as early as the beginning of the second trimester in one case.

Loss of Serum Titre Post-Partum

The other interesting observation made in the study of primiparous ewes was the observation that one of the two positive sheep bled post-partum showed a loss of titre from 1/4 to Neat. Vives et al. (1976) and Tongio and Mayer (1977) have reported that there is a decline in the reaction/

reaction frequency of positive sera taken from parous women in the last trimester of pregnancy. These results may reflect a loss of titre due to the transmission of maternal immunoglobulin to the foetus.

Source of Immunising Stimulus

Results obtained here also imply that paternally-inherited foetal antigens are the immunising stimulus in sheep. This was not directly proved but it was shown that these antibodies are not directed against maternal antigens and that they do react with paternal lymphocytes. In humans, the specificity of the pregnancy-produced antibodies has been shown by family studies. These have shown that the mother is negative, the father is positive and a proportion of the offspring are positive (Payne, 1962). The conclusion drawn is that the antigens of the foetus which are responsible for the maternal immunisation are controlled by paternally inherited genes. Antigens controlled by these genes are present in both the father and the offspring and are therefore not specific to foetal life. Unfortunately, this method is not generally applicable in sheep. Sheep are usually mated to different rams in successive matings. If antibody from a multiparous ewe did not react with the current ram, the specificity would be assumed to have arisen from a previous pregnancy. As the previous sires are seldom available, this method provides no critical evaluation of the hypothesis. Primiparous ewes with their offspring and relevant ram would provide a suitable group for testing. Unfortunately, insufficient single pregnancy families were available for these studies.

In mice, the possibility that the appearance of antibody was due to alloimmunisation by sperm has been excluded by sterile matings (Kaliss

and/

and Darg, 1964; Goodlin and Herzenberg, 1964). Similarly in humans, sperm is excluded by the observations of Terasaki et al. (1970) on a group of twenty nulliparous women. Despite the fact that the majority of the women had been exposed, none had detectable cytotoxic anti-leucocyte antibody. In sheep, this possibility has not been formally excluded. However, in the serial study of thirty-four primiparous ewes, the four responders did not develop antibody until 55 to 111 days post-coitus. In sheep, viable sperm are not found in the reproductive tract after approximately 48 hours (Hogarth, 1978). This observation argues against these antibodies being produced as a response to alloimmunisation by sperm.

Given that a proportion of parous females appear to mount an immune response against their offspring, the problem then becomes - why is the proportion not a much higher one? There are two alternative hypotheses. Firstly, the non-responders may be incapable of responding however high the dose of antigen. Secondly, there may be no or insufficient antigenic stimulus in the non-responders. From my results, I am unable to decide between these hypotheses. In the human situation, Goodfellow et al. (1976) have argued that it is unlikely that the non-responders are immunologically incompetent to respond. They quote others who have shown that maternal lymphocytes respond normally to mitogens and allogeneic tissues such as cord blood lymphocytes (Carr et al., 1974) and skin grafts (Billingham and Lampkin, 1957). However, while these results exclude generalised immunosuppression, they do not exclude the possibility of a specific immunosuppression to MHC antigens.

The alternative hypothesis is that there is an inadequate stimulus in the non-responders. In sheep, there is no evidence in favour of any particular tissue being the immunogenic stimulus. In humans, the evidence appears to favour two tissues. These are the trophoblast and white blood cells.

Goodfellow et al. (1976) looked at the expression of HLA antigens on placental membranes by the inhibition of cytotoxicity in a flourochromatic assay. They excluded the possibility that these results were caused by contamination with blood by assaying for the presence of haemoglobin. Contamination with maternal cells was excluded by comparing the expression of paternally-derived with maternally-derived HLA antigens. They concluded that placental membranes expressed foetal HLA antigens but only at about 5% of the total expressed on human spleen lymphocytes (per mg. of membrane protein). They were unable to distinguish between the possibilities that all placental tissues express low levels of antigen, or alternatively, that some subpopulation(s) of placental cells expressed more antigen than others. In particular, they could not exclude the possibility that 'the major portion of the A and B antigen activities of placental plasma membrane is attributable to the presence of surface membrane from fibroblast, blood capillary wall and other non-trophoblastic cells which should express, more or less, normal levels of A and B antigens. They went on to say "it is thus possible that the syncytialtrophoblast which forms the foetal-maternal junction lacks A and B antigens".

Faulk and Temple (1976) used an antibody peroxidase technique for light and electron microscopy. They concluded that the human trophoblast lacks B_2-microglobulin and HLA. However there was staining within/

within chorionic villi. These results favour the latter interpretation of Goodfellow et al., that only a subpopulation of cells carry HLA antigens.

Faulk & Temple and Goodfellow et al. argued that if the trophoblastic basement membrane lacks HLA antigen, the foetus is not exposed to HLA antigens from this source. However, Doughty and Gelsthorpe (1974, 1976) have eluted anti-lymphocyte antibody from placental material in humans. If the placenta absorbs out antibody activity during pregnancy, as Doughty and Gelsthorpe believe, then antigens must be present. It seems reasonable to argue from this that if there is sufficient antigen present from absorption, then there is also sufficient antigen to provoke an immune response. Although, it is possible to argue that antigen may be present but it is so presented as to be insufficiently immunogenic.

It is possible to reconcile the apparently contradictory findings of Goodfellow et al. and Faulk and Temple on one hand, with those of Doughty and Gelsthorpe on the other. In the placenta there are likely to be many areas of erosion where certain tissue layers have broken down (J. B. Solomon, 1977 - personal communication). It is possible that antigen is exposed only at these areas. Consequently, the trophoblastic basement membrane could lack antigen yet the placenta could be antigenic.

In the sheep, which has the syndesmo-chorial type of placentation, there are two layers of maternal tissue between the maternal circulation and the foetal trophoblast. It is perhaps less likely that maternal lymphocytes would be primed quite so easily in this system./

system. However, once again the areas of erosion may allow sufficient antigen-lymphocyte contact. This of course assumes that the anti-lymphocyte antibodies produced in sheep pregnancy are directed against a putative sheep MHC and that these MHC antigens are similarly distributed on sheep tissues as on human tissues.

However, an alternative explanation accepts that antigen is not present on the exposed trophoblast. Johnson (1974) has argued that antigen trapping could take place within the placenta as antibody passes through to the foetus. This however would not work for sheep where antibody transfer does not occur pre-partum.

Alternatively, the antigen stimulus could come from foetal white blood cells in the maternal circulation. Again there are no data available for sheep and I will base my arguments on evidence obtained in the human. That foetal leucocytes do occur in maternal blood has been shown by Schroder and de le Chapelle (1972) who detected a low frequency of cells carrying Y chromosomes in metaphase spreads of maternal white blood cells. Subsequently, Herzenberg et al. (1979) were able to detect the presence of HLA-A2 positive blood cells in HLA-A2 negative mothers using a Flourescent Activated Cell Sorter.

The source of these foetal white blood cells may be the leakage of foetal blood into the maternal circulation. Cohen et al. (1964) have shown in a careful study of 622 women that the intermittent entry of foetal erythrocytes into the maternal bloodstream is a common occurrence. Post-partum, foetal erythrocytes were detected in 43 of the 89 cases studied.

Schroder and de la Chapelle (1972) noted that the frequency of foetal lymphocytes in the mother's blood was much higher than expected compared with the frequency of foetal red blood cells. They argued from this that foetal lymphocytes may actively cross the placenta. The evidence is based on a small sample and they admit that the technique they use for detecting foetal white blood cells is not too reliable. Nevertheless the argument is plausible. Lymphocytes are known to migrate through endothelial cells in post capillary venules in the lymph nodes (Gowans & Knight, 1964).

The time of appearance of cytotoxic anti-leucocyte antibodies in humans and sheep is not really helpful in deciding between trophoblast or foetal white blood cell immunisation. The early occurrence of antibody in the study of Van der Werf (1971) led him to suggest that in certain cases the trophoblast may provide the source of immunising antigen. Antibody was detected in one case at $6\frac{1}{2}$ weeks and in three cases at 12 weeks of gestation. In human embryos, granulocytes have not been detected until 15 weeks, while lymphocytes do not occur until three to four weeks later. This is too late to account for the observed appearance of antibody.

The earliest date at which I could detect antibody was at 55 days of gestation. Solomon (1970) has reported detecting lymphocytes in foetal sheep at 40 days of gestation. Consequently, the antibody appearance could be elicted by immunisation with either trophoblast or foetal white blood cells.

In summary, the evidence for foetal trophoblast or for foetal leucocytes being the antigenic source for the production of anti-leucocyte antibodies in both humans and sheep is not conclusive.

Therefore it seems speculative to decide if the non-responders fail to respond because of a lack of adequate stimulus or because of immunoincompetence.

Antilymphocyte Antibodies and Foetal Abnormalities

The final problem I wish to consider in the discussion is the association between these antibodies and foetal abnormalities. Terasaki et al. (1970) have reported a slight association between the incidence of HLA antibodies and congenital anomalies. Subsequent work has not confirmed this association. (Naito et al. 1970; Sever and Terasaki, 1970; Harris and Lordon, 1976). Jensen and Kissmeyer-Nielson (1969) have suggested that any association may be due to malformed embryos being more likely to induce cytotoxic antibody. I have not specifically looked for any association with foetal abnormalities and the presence of cytotoxic anti-lymphocyte antibodies in sheep. Nonetheless, it is quite certain that the vast majority of sheep which possess pregnancy-induced antibodies give rise to normal offspring.

Any immunity to the effects of antibody would appear to be specific to anti-MHC antibodies, at least in the human, since antibodies directed at other antigens are known to result in clinical syndromes. This is true for antibodies directed against red blood cells (Levine et al. 1941 a,b) platelets (Jones et al. 1961) and neutrophils (Hitzig and Gitzelmann, 1959; Lalezari et al., 1959, 1960). Two hypotheses can be put forward to account for the absence of damage. Firstly, the antibodies may be absorbed out before they reach the foetus. Alternatively, the antibodies may react with the foetus but not cause any damage.

There are two possible sites of absorption. The first is the maternal circulation. Absorption may be by soluble antigen or by foetal cells present in the maternal circulation. Tung (1974) has reported the presence of circulating antigen-antibody complexes in pregnant mice and guinea pigs. However, the fact that antibody is nonetheless detectable in maternal serum would argue that this is not the complete explanation.

The second site of absorption is at the placenta. Once again the presence of antibody in the maternal serum implies that this method is not entirely efficient. However, it may be that the placenta removes all antibody which comes near it and prevents any reaching the foetus. Antibody remaining in the maternal circulation would, according to this hypothesis, represent residual antibody which had not passed by the placenta. Maternal immunoglobulins on placenta have been detected by immunohistological methods in humans (McCormick et al., 1971) and mice (Voison and Chaouat, 1974). The elution studies of Doughty and Gelsthorpe (1974, 1976) and Revillard et al. (1976) argue in favour of this hypothesis.

Anti-Ia antibodies are known to occur in human pregnancy sera (Winchester et al.,1975; Thompson et al.,1976, Ferrone et al., 1976). However, Goodfellow et al. (1976) found no evidence for Ia antigens in human placenta. This would suggest that the placenta does not act as a filter for these antibodies. Nonetheless, Revillard et al. (1976) managed to elute from placentae a population of immunoglobulin, which could not be absorbed by platelets and were cytotoxic for B but not for T lymphocytes. They inhibited MLR and mitogenic stimulation of lymphocytes and the Fab_2 fragments prevented EA rosette formation/

formation with Fc receptor bearing lymphocytes. These properties strongly suggest anti-Ia antibodies. If the placenta is the sole antigenic stimulus, it would need to be Ia antigen positive to provoke anti-Ia antibody formation. If one accepts that the foetus is prevented from harm by the placenta absorbing out cytotoxic anti-MHC antibodies, the problem of foetal damage is not solved. It merely shifts from "why is the foetus not damaged?" to "why is the placenta not damaged?".

The other explanation put forward for the absence of damage is that the antibodies do come into contact with cells inside the foetus but they do not cause any damage. Tiilikainen et al. (1976) and Harris and Lordon (1976) experienced some difficulty in detecting paternal but not maternal HLA specificities on neonatal and cord blood lymphocytes. This could be due to the masking of antigen by specific antibody, i.e. the antibody binds to the antigen but is without effect.

The reasons for the absence of effect of the antibody are not known with certainty. HLA antigen is thought to occur on most nucleated cells in the human. Jensen and Kissmeyer-Nielsen (1969) have suggested that antibody will be dispersed among many cells. This dilution will prevent any deleterious effect.

Tiilikainen et al. (1976) have suggested other explanations which might be applicable. These include coating of antigen with non-cytotoxic antibody, antigen shedding, pinocytosis and antigenic modulation.

The observation made earlier, that anti-MHC antibodies are non-toxic while/

while anti-red blood cell, anti-neutrophil and anti-platelet antibodies are, would be compatible with both the placental absorption and antibody dilution theories. It is less easy to understand how absorption by foetal cells in the maternal circulation or the suggestions of Tiilikainen et al. (1976) could explain these differences.

In sheep, there is no evidence for or against any hypotheses. In this animal, antibody transfer to the offspring takes place only post-partum via the colostrum (reviewed by Brambell, 1970). It should be possible to decide if specific anti-foetal antibody is absorbed before it reached the foetus by examining the colostrum of antibody positive primiparous ewes. If anti-lymphocyte antibody is found in this colostrum, this would favour the interpretations of Jensen and Kissmeyer-Nielsen and of Tiilikainen et al.. If it is not found, this would favour absorption by placenta or by antigen or cells of foetal origin. However, the presence of antibody in maternal serum, but its absence in colostrum, is not conclusive. Certain classes of immunoglobulin are selectively transferred to the colostrum. The antibody could be of the wrong immunoglobulin class.

B. Antisera Produced by Deliberate Immunisation

The use of parous sera involves screening a large number of samples to find sera which are suitable with regard to titre and specificity. Many workers investigating species with no ethical barriers to immunisation, prefer to immunise prospective serum donors to raise suitable antisera. Alloimmunisation is routinely preferred to xenogeneic/

xenogeneic immunisations due to the frequent occurrence of species specific antigen in inter-species immunisations. Among the advantages of deliberate alloimmunisation procedures are a) high titre antisera, b) use of animals of choice, not only from the scientific side, e.g. animals with interesting, rare or null alleles as donors, but also those convenient to husbandry arrangements, c) the existence of a single donor simplifies absorption analysis and d) the type of immunisation may provide other information of interest, e.g. organ allograft survival and tissue distribution of shared antigens.

However, others have preferred to make use of parous sera to define lymphocyte alloantigens, either exclusively or in conjunction with sera from deliberate immunisations. Many parous sera are perfectly adequate for tissue typing, and the acquisition of parous sera involves less labour and fewer skilled techniques. Additionally, it is just possible that induced as opposed to natural immunisation may not select the most important antigenic determinants in the normal host.

Many authors have shown that major histocompatibility alloantigens are present on many, if not all, nucleated cells in mice and men. This probably holds for most other higher vertebrates also (reviewed in Chapter 1). This being the case, it is probable that a variety of tissues can be used to raise antisera to lymphocyte alloantigens. Certainly, a variety of procedures have been successfully used to raise antisera in different species. In man, several authors have reported that multiple transfusions can produce anti-leucocyte antibodies/

antibodies (Payne, 1957; van Loghem et al., 1958; Oh et al., 1972). Ferrara et al. (1972, 1978b) have shown the same effect with repeated transfusions of small amounts of blood. Skin grafting has also been used both in man (Batchelor, 1965) and in other species including dogs (Vriesendorp et al., 1971), rhesus monkeys (Balner et al., 1965b, 1967), sheep (Ford, 1973), cattle (Spooner et al., 1979) and pigs (Vaiman et al., 1970b). Other methods which have produced antisera have included small bowel transplants in dogs (Vriesendorp et al., 1971) and the use of spleen acetone powder in dogs (Léon et al., 1975 a,b). The intravenous, subcutaneous or intramuscular injection of purified white cells has also been used successfully both on its own (Shanbrom et al., 1968; Payne et al., 1970) and in combination with skin grafting (Walford et al., 1965; Walford and Troup, 1967; Payne et al., 1967; Thorsby and Kissmeyer-Nielsen, 1969).

Two methods were attempted in sheep to raise anti-lymphocyte sera. These were skin grafting and the injection of purified white cell preparations.

Materials and Methods

1. Lymphocyte Immunisation

Lymphocyte Immunisations

Preparation of Cell Suspensions for Injection

500 mls. of whole blood were taken by jugular venepuncture into evacuated glass bottles with EDTA (pH 7.0 final concentration 0.02M).

Whole blood was then centrifuged in 250 ml. aliquots for 60 minutes at 1,600 g. The buffy coat was removed and washed twice in phosphate buffered saline (pH 7.2) + 1 g/litre glucose, for 30 minutes at 1,000 g. The purified white cell suspension was then resuspended in 10 mls. Hank's Balanced Salt Solution + 10% non-reactive pooled rams' serum + 100 i.u. penicillin + 100 ug. streptomycin/ml. without sodium bicarbonate. These cell suspensions were stored at 4^{o}C. in the dark.

Dose Administration

The cell suspension was divided into three equal amounts which were given, 2, 9 and 16 days after bleeding the cell donor, to the recipient. Cell suspensions were given intramuscularly in the hind leg.

Sheep

A trial test was performed on three pairs of sheep. Two pairs were sire-offspring combinations. These four sheep were of the Scottish Blackface breed. The third pair were full-sibling Finnish Landrace x Dorset Horn (FL x DH) ewes.

After this, twenty-one full-sibling pairs of nulliparous Finnish Landrace x Dorset Horn lambs were used. In one pair, only one animal acted as a donor, the second animal of the pair acting as the recipient.

Serum Samples

Serum samples were taken on the day of injection and 2, 9, 16, 21 and 28 days after injection. All serum samples were tested for cytotoxicity against the lymphocytes of the cell donor in a type I mlct. These samples were all tested at two concentrations undiluted and 1/8. Positive sera were subsequently tested for specificity against a panel of 41 sheep.

Pre-formed Cytotoxic Antibody

All animals with one exception were nulliparous and contained no pre-formed cytotoxic antibody in their serum. The exception was 5L2093 which had given birth to two animals at its one previous lambing. It contained an apparently monospecific antibody with a low reaction frequency (<5%) at a titre of 1/8. This antiserum was non-reactive with the lymphocyte donor.

Skin Grafting

Grafting Procedures

Sterile procedures were observed throughout. All sheep were starved overnight prior to skin grafting. Pairs of sheep were brought in and anaesthetised with 5% halothane in a 50:50 N_2O:O_2 mixture. A large area of wool behind the front elbow was dipped, washed and disinfected with hibitane. Two pieces of skin, both approximately 1 cm. square, were cut from each animal. The upper graft was stitched into the lower bed. This autograft acted as a control. The lower graft was stitched into the top bed of the other animal. The skin sections were dressed with vaseline gauze,

a/

a gauze swab and a polystyrene foam pressure pad held in position by a tight crepe bandage around the body.

Sheep

Initially, reciprocal grafts were exchanged between three pairs of parous ewes from three breeds. Two pairs of animals were paternal half-sibs born in the same year. The Finnish Landrace x Dorset Horn pair were four years old. The pair of Border Leicester ewes were three years old. The third pair were full-sibs born in successive lambings. These were two Southdown sheep, three and four years old respectively.

Serum Samples

Serum samples were taken on the day of grafting and 22, 35, 42 and 56 days after grafting.

Cytotoxicity Testing

All serum samples were tested at two concentrations (neat and 1/8) in a type I mlct. The samples were tested against a panel of forty sheep which included the graft donor and recipient.

Pre-formed Antibodies

Of the six serum samples taken prior to first grafting, two contained pre-formed antibody. Animal 4S1 gave only partial reactions at neat only and was negative at 1/2. Animal 5S22 had cytotoxic anti-lymphocyte antibodies to a titre of 1/8. These two animals were full siblings and were used to exchange reciprocal grafts. The sera were non-reactive with each other. The other four sera were all negative.

Booster Immunisations

Four animals, 4FD3, 4FD15, 4S1 and 5S22 were boosted with a second exchange of reciprocal grafts from the same donors. Grafts were re-exchanged 124 days after the first exchange. Serum samples were taken on the day of grafting and after 7 and 16 days.

The other pair of sheep, 5L17 and 5L22, received reciprocal injections of a suspension of the other's lymphocytes suspended in Freund's Incomplete Adjuvant. Serum samples were taken immediately before injection and after 8 and 40 days.

Results

1. Lymphocyte Immunisations

A total of forty-seven sheep received intramuscular injections of allogeneic lymphocytes. One animal received but did not donate lymphocytes. It responded by producing a high titre (1/128), highly reactive serum. The other forty-six sheep donated and received lymphocytes between related pairs. Two pairs were ram and offspring sets. Both rams and one offspring produced antibody. One set of lymphocyte injections was exchanged between a pair of full sibling ewes. One animal, which had lambed previously, already possessed antibody in its serum. The specificity and titre of the antibody showed no change after immunisation. This animal has been counted as a non-responder. The other ewe produced a high titre, highly reactive serum. The remaining twenty pairs of sheep were full-sibling lambs. Eighteen out of the forty sheep produced antibody. In five pairs both animals produced antibody. In eight pairs only one animal produced antibody. In seven pairs neither lambs produced antibody.

Results presented later (Chapter 6) suggest that lymphocyte antigens in sheep are controlled by a single genetic system. If this is the case, even if both parents are heterozygous for different alleles, one quarter of pairs of full-siblings will be identical. Identical full-siblings are not expected to produce antibody. The occurrence of homozygous parents or the presence of shared haplotypes between parents will increase the expected proportion of non-responders. It seems probable, therefore, that some sheep failed to produce antibody because of absence of antigenic disparity. Monozygotic twinning or chimaerism will also increase the expected proportion of non-responders. However, both phenomena are rare in sheep (Stormont et al., 1953).

The numbers of responders and non-responders and the serum titres of the responders are given in figure 1. It can readily be seen that lymphocyte immunisations provide a suitable method for the production of antisera to sheep lymphocytes. This is in agreement with results obtained by Millot (1978) and by Schmid et al. (1975).

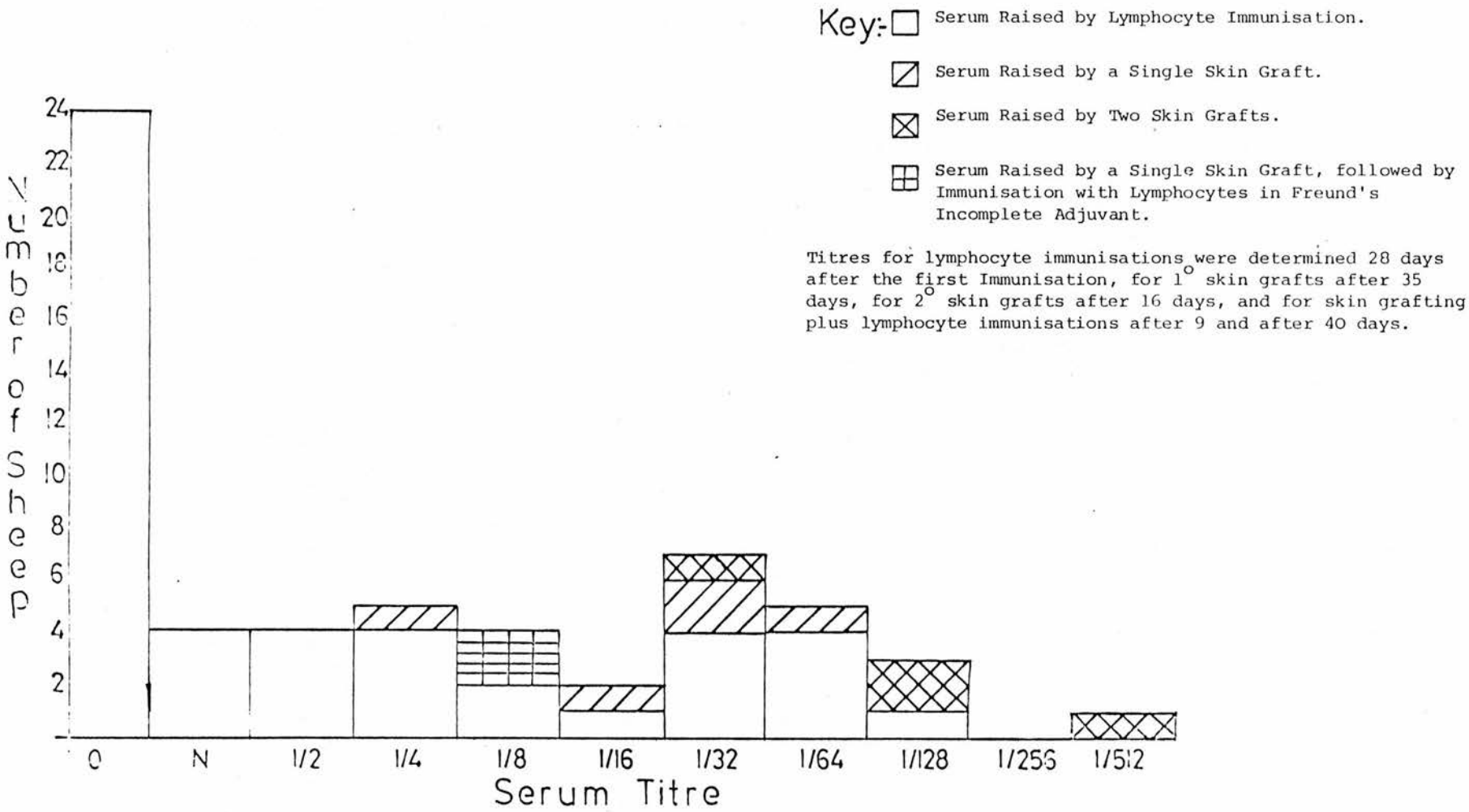
2. Skin Grafting

Six animals received skin grafts. One animal failed to produce antibody. The other five animals all responded to the skin graft. The two animals which had pre-formed antibody both showed an increase in titre and reaction frequency. Results are given in figure 1.

Four sheep were given a second skin graft. All showed an increase in titre over the levels obtained immediately prior to grafting. One animal had not responded to the first graft. This animal produced a high titre (1/128) serum to the second graft.

Figure 1

The Titres of Sera Raised by Deliberate Alloimmunisation



Two animals were given a booster injection of lymphocytes in Freund's Incomplete Adjuvant. Both animals showed no change in either serum titre or reaction frequency.

This study is small but it does suggest that skin grafting is an excellent procedure for the production of high titre antisera in sheep. This is in agreement with results obtained by Ford (1973) and by Schmid et al. (1975). Injection of lymphocytes in Freund's Incomplete Adjuvant does not appear to be very successful.

3. Foreign Sera

There have been three previous studies on sheep leucocyte antigens, Millot (1978) used nine sera to detect eleven antigens in French prealpe sheep, Schmid et al. (1975) used thirty-one sera to detect thirty-one antigens in German Merino Landrace sheep. Ford (1975) used twenty-two antisera to detect twenty-two antigens in a variety of breeds in England. Drs. Millot, Ford and Cwik kindly agreed to send me samples of their sera. Drs. Millot and Ford sent me samples of sera as used. The sera used in the study by Schmid et al. (1975) had been absorbed to decrease the reaction frequency of the sera. Unfortunately, absorbed sera were not available so the samples received were unabsorbed sera.

All sera were titrated against a panel of forty sheep. Test dilutions started at a dilution of 1/4. This was for two purposes. Firstly, to conserve antisera. Secondly, if a positive serum does not react at a dilution of 1/4, the serum will possibly be too low titre for reliable testing. Detailed results are given in Table 7.

Table 7

Titres of Foreign Sera

Serum Identity	Source	Breed of Origin	Type	Titre
Anti - 1	France	NK	L	1/1024
Anti - 2 + 9	France	P	L	1/16
Anti - 2 + 10	France	P	L	1/1024
Anti - 3	France	P	L	1/128
Anti - 4	France	P	L	1/16
Anti - 5	France	P	L	1/256
Anti - 6	France	P	L	1/64
Anti - 7 + 11	France	P	L	1/1024
Anti - 8	France	P	L	1/128
9102 Re	West Germany	GML	L	1/32
9103	West Germany	GML	L	1/64
9103 Re	West Germany	GML	L	1/32
9104	West Germany	GML	L	1/64
9104 Re	West Germany	GML	L	1/128
9107	West Germany	GML	L	1/32
9108	West Germany	GML	L	NRP
9109	West Germany	GML	L	1/4
9110	West Germany	GML	L	1/16
9111 Re	West Germany	GML	L	1/4
9112 Re	West Germany	GML	L	NRP
9113	West Germany	GML	L	1/64
9114	West Germany	GML	L	1/8
9115	West Germany	GML	L	1/8
9116	West Germany	GML	L	1/64
9117	West Germany	GML	L	N.T.
9501	West Germany	GML	L	1/32
9502	West Germany	GML	L	1/32
9503	West Germany	GML	L	1/8
9506	West Germany	GML	L	NRP
9507	West Germany	GML	L	1/32
9509	West Germany	GML	L	1/8
9511	West Germany	GML	L	1/8
9512	West Germany	GML	L	NRP
9703	West Germany	GML	L	1/256
9704 Re	West Germany	GML	L	NRP
9705	West Germany	GML	L	1/64
9706	West Germany	GML	L	1/64
9706 Re	West Germany	GML	L	1/64
9707	West Germany	GML	L	1/16
9710 Re	West Germany	GML	L	1/4
9711	West Germany	GML	L	1/32
9711 Re	West Germany	GML	L	1/64
9712 Re	West Germany	GML	L	NRP
9715	West Germany	GML	L	1/32
9716	West Germany	GML	L	1/64
9751	West Germany	GML	S	1/4
9752	West Germany	GML	S	1/32

Serum Identity	Source	Breed of Origin	Type	Titre
9753	West Germany	GML	S	1/32
9754	West Germany	GML	S	NRP
9755	West Germany	GML	S	1/32
9758	West Germany	GML	S	1/64
66/0	England	CF	P	1/4
132/0	England	CF	P	1/4
138/0	England	CF	P	NRP
120/0	England	CF	P	1/4
80/1	England	CF	MOA	1/4
151/0	England	CF	P	1/2
257/0	England	CF	P	N
282/0	England	CF	P	1/4
90B/0	England	RM	P	N
148/4	England	CF	MOA	1/4
140/1	England	CF	MOA	1/8
141/7	England	CF	MOA	1/8
598/1	England	CF	MOA	N
163/10	England	CF	MOA	NRP
88B/1	England	RM	SOA	NRP
206/0	England	CF	P	NRP
87B/0	England	RM	P	NRP
81B/7	England	RM	SOA	1/16
260/2	England	RM	SOA	N
188/2	England	SCBL	S	1/2
N269/0	England	CF	P	NRP
C22/0	England	FL	P	NRP

Key:

1. Breeds of Origin -
   - NK = Not known
   - P = Preape
   - GML = German Merino Landrace
   - CF = Clun Forest
   - RM = Romney Marsh
   - SCBL = (Suffolk x Cheviot) x Border Leicester
   - FL = Finnish Landrace

2. Type -
   - L = Antiserum raised by Lymphocyte Immunisation
   - S = Antiserum raised by Skin Grafting
   - P = Antiserum obtained from a Parous ewe
   - MOA = Antiserum obtained from recipient of a massive osteoarticular allograft
   - SOA = Antiserum obtained from recipient of a small osteoarticular allograft

3. Titre -
   - NRP = Non-reactive at a dilution of 1/4 against a panel of at least forty sheep

Several sera were selected for more detailed analysis. This is discussed in the next section.

4. Selection of Sera for Use as Typing Reagents

Before being used as typing reagents, all sera were titrated to determine the end-point. Reagents for typing were used two doubling dilutions before the end-point. There were three reasons for using diluted sera. Firstly, to improve reproducibility. Sera used at or near the end-point give variable reactions with a high proportion of weak positives. It was felt that by not using weak sera, test reproducibility would be improved. Secondly, many sera showed a reduced reaction frequency when used at the test dilution. This was probably due to the presence of several antibody populations in a serum. Using titrated sera which detected fewer antigens would ease the subsequent analysis. Thirdly, when using clusters of sera to define antigens, sera which only react with part of the cluster can cause considerable complications. They are either due to genuine 'splits' in the antigen assignments or alternatively to the presence of weak antisera. By using titrated sera, I hoped to reduce the incidence of weak antisera.

Sera were titrated against a panel of forty heterogenous sheep from several breeds. Only some sheep reacted with any given serum. I used a similar panel of positive and negative sheep to titrate all sera. By carrying out titrations against positive and negative sheep, this gave an indication of the number of specificities present in a serum and also an indication of the usefulness of the serum as a typing reagent.

The titres of parous sera ranged from 1/1 to 1/256. The majority of sera fell in the range 1/1 to 1/64. The titres of sera raised by deliberate immunisation ranged from 1/1 to 1/1024. In general, immune sera had higher average titres than parous sera.

Four sera, which were undoubtedly positive in previous tests against different sheep, could not be titrated. They failed to react with any of the sheep used for titrations. This is probably due to either the very low titre of these sera or alternatively to the test panel lacking specificities. The four sera appeared to detect three specificities. Two sera detected one specificity, while the other two sera detected the others. These sera were included in the subsequent tests.

Several of the foreign sera also failed to react (Table 7). As titrations started at a dilution of 1/4, the absence of positive reaction could be due to the low titre of the serum, my panel lacking specificities or alternatively to different test sensitivity between my test and that used originally. The non-reactive foreign sera were not used in subsequent tests.

All sera which did react were titrated. All sera used for testing had a minimum titre of 1/4. This was to ensure that each serum could be used at least two dilutions above the end-point. Also, wherever possible, sera which had a consistent reaction frequency at two or more dilutions were chosen. This was to ensure that sera used in my tests gave reliable results.

112 sera were finally chosen for subsequent analysis. The source of the sera is given in Table 2 of Chapter 5. Not all of the reactive/

reactive foreign sera were used. Titrations revealed the presence of several antibody populations in many sera. Several antibody populations were expected in the sera from W. Germany. The sera tested here are unabsorbed samples of the sera used by Schmid et al. (1975). However, most of the sera supplied by Ford and Millot were thought to be monospecific (Ford, 1974; Millot, 1978). The presence of extra antibodies, as shown by titrations, may be due to different test sensitivities. Many of the titres found by me are very much higher than those reported for the same sera by Ford (1973) or by Millot (1978). It is probable that my test is more sensitive than the tests used by Ford and Millot. It is possible therefore that I am detecting extra antibodies which went undetected by Ford and Millot.

The results obtained with the selected sera are described in Chapter 5.

CHAPTER 5

THE DETECTION OF LYMPHOCYTE ANTIGENS

Introduction

There are two stages in the identification of antigen systems. The first is the resolution of individual antigens. The second stage is the elucidation of the relationships between them. In this chapter, I will deal only with the detection of specific antigens. The relationships between them will be discussed in the next chapter.

There are also two steps in detecting antigens. The first involves selection of a suitable test system. It is necessary for the test to be of sufficient sensitivity and of high reproducibility. Further, the test must be of convenient format for large scale testing. The test used here, and for most white blood antigens is the microlymphocytotoxicity test. This has been more fully discussed in Chapter 3. The second step in antigen detection involves the acquisition of suitable antisera. Generally, the aim is to produce sera which detect the products of single antigenic specificities. There are two models for the production and detection of monospecific sera. The first model is the approach used in human red blood cell grouping. The procedure here is to determine the specificities in a serum by extensive cross-absorptions. Once the specificities have been identified, the sera can be used to produce one or more reagents specific for individual antigens. The other antibodies in the serum can be removed by the appropriate absorptions. This approach is admirably explained by Race and Sanger (1975).

However, Bodmer and Payne (1965) have pointed out two possible pitfalls in the cross-absorption procedure. They state that the antibodies in a serum will not be resolved -

(a)/

(a) if all cells used for absorption carry all the antigens detected by the serum, or

(b) if all test cells, and hence all cells used for absorption, all lack some of the specificities detected by the serum.

Bodmer and Payne (1965) illustrate the problem with an example. The serum contains two antibodies anti-A and anti-B. A and B are two hypothetical antigens. The two antigens can occur in all four possible combinations, AB, A, B and - (null). They set U, V and W to be the relative proportions amongst the positive cells of the combinations A, B and AB where $U + V + W = 1$. Further, they set m to equal the number of cells typed with the absorbed sera. The number of cells used for absorptions is n. The probability that all cells used for absorption are AB (i.e. contain all antigens detected by the antiserum) is W^n. The probability that all cells are A is U^m. The probability that all test cells are B is V^m. Therefore the total probability of not resolving the antibodies in the serum is $U^m + V^m + W^n$. If one of the specificities (say B) is rare, then U^m (the probability that all cells are A) will be very much greater than $V^m + W^n$ (the probability that all cells are B plus the probability that all absorbing cells are AB). Therefore, in this case, the procedure is to minimise U^m. This can be done by increasing the size of the test panel (m). Alternatively, one can try to minimise the size of U. This point is discussed later.

Walford et al. (1967) have discussed the problem caused by the possibility that all cells used for absorption carry all the antigens detected by the serum. They point out that, if the donor is used in the test panel, at least one of the test cells will carry all the antigens./

antigens. Therefore, the problem posed is to minimise the probability W^n. Again, they use the example of a serum containing antibodies against hypothetical antigens A and B. They ask "at the 5% probability level how many absorptions with positive cells followed by the demonstration of negative reactions on back testing against cells of the original immunising donor are required before one can presume that the serum is either monospecific or detects factors associated at least 90% of the time?". By setting $W = 0.9$ and $P<0.05$, the equation $P = W^n$ is solved when $n>29$. It is of course possible to set different values for P and W. Different values could produce different values of n. In general it is not possible to prove monospecificity by cross-absorptions. It is only possible to make it extremely likely. The convention has therefore arisen of calling white blood cell sera "operationally monospecific". By the criteria of Walford et al. (1967) a sera is called "operationally monospecific" if no more than one antibody is detected in thirty or more absorptions. This definition has been widely accepted. Unfortunately, technical problems with white blood cell absorptions have often prevented the practical application.

Dausset et al. (1965) have pointed out that antigens which are associated in one population need not be associated in another. They recommend the use of heterogeneous cell panels drawn from different populations for screening and testing sera. As the frequency of antigens may vary in different populations, the use of heterogeneous panels for absorption is also expected to reduce the possibility that all absorbing cells will be of one antigen type.

If monospecific sera are used, antigens can be detected by the reactions of single sera. The probability of false negatives and of false positives depends upon the reproducibility of the test procedures.

The alternative procedure for detecting antigens involves the use of groups of multispecific sera. Each group must contain sera which all detect a common determinant (say A). Each serum may detect other non-A antigens as well. If an individual reacts with all the sera in a group, it is probable that he possesses the common determinant. It is possible that an individual could react with all the sera in a group but not possess the common determinant. The probability of this is given by (1-a) Πb; where a is the frequency of the common (A) determinant and b is the frequency of the non-A reactions. The symbol Π is the multiplication product of the non-A frequencies in each serum. As the number of sera in the group increases the product of the 'tail' frequencies tends to zero. In practice, if a group of associated multispecific sera divides animals into two groups according to whether the animal reacts with most of the sera in a group, then the group of sera can be used to define an antigen.

The problem with using multispecific sera for analysis is, of course, deciding when sera share a common antigen. This is usually, but not always, accomplished by comparing sera in pairs. Each serum is compared with every other serum. A two-by-two table is constructed for each serum pair. The significance of the association can be calculated exactly by Fisher's Exact Test (Fisher 1946) or approximately by calculating the contingency chi-square value (Fisher 1946).

Due to the comparative simplicity of calculation the chi-square is usually used. The correlation between two sera can be calculated by $r = (X^2/N)^{\frac{1}{2}}$. (Dausset et al., 1965). The correlation measures the magnitude of the association between two sera. This correlation varies between -1 and 1. If the number of discordant reactions is greater than the number of concordant reactions, r is given a negative value. As the frequency of the unshared reactions tends to 0, r tends to 1. Significant associations between two sera may be due to a shared antibody. Alternatively, the sera may detect antibodies directed against antigens which are associated in the population. Distinguishing between associations due to shared antibodies and associations due to antibodies directed against associated antigens is one of the main difficulties of this approach. (For a fuller discussion see Bodmer et al., 1969). Other statistical methods also exist for detecting the presence of shared antibodies in multispecific sera (e.g. Selwood, 1972).

If groups of multispecific sera are used to define antigens, then false positives are due to animals reacting with the tails of all or nearly all of the sera. If the tails show significant associations with other tails this problem is pronounced. The frequency of false positives can be reduced by increasing the number of sera or alternatively, by reducing the incidence of 'tail' reactions. The length of the 'tail' can be reduced by appropriate absorptions or by using diluted sera. False negatives are due to sera failing to react although they have the antibody and the animal has the relevant antigen. This probably represents irreproducibility of the technique./

technique. The effect of false negatives can be reduced by increasing the number of sera used to define a cluster.

Once antigens have been defined, it is usual to look at the inheritance patterns in families. If inheritance follows a simple mendelian pattern, this is generally taken as confirmation of the antigen definition. Also, titration of a serum can indicate the presence of only one antibody population. This too can be a valuable aid to assessing the specificities present in a serum. The latter method is probably insufficient on its own to indicate the presence of a single antibody population but it can be helpful in conjunction with other methods.

Materials and Methods

1. Selection of Sera

After titrations had been completed the most interesting sera were selected for further study (Chapter 4). No serum with a titre determined as being less than 1/4 was used in the subsequent analysis. Sera with a low proportion of strong positives and very few weak positives were chosen. All titrated sera were used two doubling dilutions below the end point. A number of sera could not be titrated. Although they were tested against cells from thirty-six or more different animals they failed to give any positive reactions. As their original reactions in the screening procedure were very promising, four of these untitrated sera were included in the subsequent analysis. They are indicated in the text by an asterisk (e.g. 016*)./

(e.g. 016*). Ultimately, 112 sera were chosen for further analysis. They are listed in Table 2. The 112 sera came from 94 different animals from four different countries. The breed of origin of the 112 sera is given in Tables 1 and 2. Twelve sera came from West Germany, one from France and seven from England. Ninety-two sera came from Scottish sheep. Seventy-four different animals provided the Scottish antisera. Fifteen donors each provided two antisera. They are indicated in the text. Four sera were tested at two different dilutions. Three came from Finn-Dorset sheep and one came from a Scottish Blackface sheep. With the exception of four English sera, the immunising stimulus for all sera came from animals of the same breed. The presence of the antibody therefore indicates that the antigen is present in the breed. The four exceptional English sera came from a Welsh Mountain, a Romney Marsh, a Clun Forest and a Suffolk x Cheviot x Border Leicester cross sheep.

2. Cell Panel

As stated earlier, the problem in using groups of associated sera to define antigens is that it is difficult to decide if a pair of sera are associated because they share antibodies or because they detect antigens which are associated in the population. To minimise the association of different antigens in a cell panel, large heterogeneous panels of unrelated animals are recommended for testing sera of unknown specificity.

My sera were tested against 258 different lymphocyte preparations from 253 different animals. Two lymphocyte preparations were made from/

Table 1

Source of Sera and Animals

Breed	Number of Animals used for Serum Production	Number of Animals used in the Test Panel
Scottish Blackface	25 (6)	-
Finnish Landrace	4 (2)	21
Damline	7	123 (2)
Border Leicester	5 (2)	9 (1)
Southdown	1	14
Welsh Mountain	3	5
Tasmanian Merino	-	5
East Friesland	-	19 (2)
Oxford Down	4 (2)	24
Dorset Horn	-	3
Cheviot	2	1
Finnish Landrace x Dorset Horn	24 (6)	1
Oxford x Texel	-	23
Tasmanian Merino x Welsh Mountain	-	5
German Merino Landrace	12	-
Prealpe	1	-
Clun Forest	4	-
Romney Marsh	1	-
Suffolk x Cheviot x Border Leicester	1	-
TOTALS	94 (18)	253 (5)

N.B.:

(1) Four sera were each tested at two dilutions. Three came from Finnish Landrace x Dorset Horn ewes. One came from a Scottish Blackface ewe.

(2) Eighteen sera were repeat bleeds from animals which had already donated antisera. These repeat bleeds are referred to in brackets. Thus, six sera came from repeat bleeds of Scottish Blackface animals. In total, twenty five different Scottish Blackface ewes provided thirty one antisera.

(3) Five animals were each tested twice. They are presented in brackets. Thus, two Damline, two East Friesland and one Border Leicester each provided two lymphocyte preparations.

from each of five animals. It was not feasible to select unrelated animals for this study. Further, many of the animals were chosen to form families to study the inheritance of the antigens. Care was taken to choose a large heterogeneous panel. The origin of the 258 lymphocyte preparations is given in Table 1.

3. Family Studies

The animals used for family studies came from three farms. Animals of the Finnish Landrace breed from the Animal Diseases Research Association farm at Moredun were kindly made available by Dr. Gardiner. The other two farms belonged to the Animal Breeding Research Organisation. The two East Friesland and five Damline families came from Skedsbush Farm. The Welsh Mountain-Tasmanian Merino cross family and the Oxford Down sheep came from Dryden Field Station at Roslin.

Care was taken to minimise false parentages. All sheep were typed for R-O-i red cell antigens, haemoglobin and high and low potassium levels. The two families from Moredun were also typed for Transferrins. The inheritance of these traits is well established (Tucker, 1975). Methods used are described below. However these traits alone will probably not pick up all false parentages. Dr. Nguyen kindly agreed to test 120 sheep against eleven sheep systems. The systems tested were R-O-i, the A system, the B system, the C system, D,M,F30,F41, C4' C4" and haemoglobin.

Unfortunately, it was not possible to send the same sheep that were being typed for lymphocyte antigens. Instead a selection of sheep from Skedsbush and from Dryden were sent. From Dryden, three rams, twenty-three/

twenty-three ewes and forty-eight lambs were sent. The tests showed that parents had been wrongly assigned to six lambs. The estimate of parentage error for Dryden is 12.5%. From Skedsbush, four rams, nineteen ewes and twenty-three lambs were sent. Here, only one animal was shown not to be the offspring of the reported parents. At Skedsbush, the estimate of parentage error is 4.3%. It must be stressed that these figures are minimum estimates of the false parentage error amongst these sheep. Probably due to a certain amount of inbreeding, many ewes and rams shared the same alleles. Therefore some false parentages could go undetected.

As a consequence of the observed high level of false parentage in sheep from Dryden farm, families from this farm were carefully selected. The Oxford family (one ram, three ewes and three lambs) was chosen because only one Oxford ram existed. Further, animals of the Oxford Down breed are readily distinguished from other breeds and crosses involving this breed. The other family (one Welsh Mountain ram, five Tasmanian Merino ewes and five lambs) was "hand mated". Special care was taken to ensure that the pedigrees were correct. The animals of this cross were easily distinguished from other sheep at Dryden. They were the only Welsh-Merino crosses on the farm and the ram and four of the offspring had black fleeces. All the Skedsbush families were Damline or East Friesland. The Damline breed is a cross-breed involving four other breeds. One of the four breeds is the East Friesland. The Dryden and Moredun sheep were used to provide a variety of breeds for family studies.

4. Blood Typing Procedures

The procedures used for potassium haemoglobin and transferrin types were those of Rasmusen et al. (1974).

Potassium type:- Whole heparinised blood diluted 1 in 201 was estimated for potassium in an EEL flame photometer. Animals with values of 40 m. equiv. K per litre of erythrocytes and below were classified as low (LK), others as high (HK).

Haemoglobin type:- This was determined by starch gel electrophoresis (Starch-Hydrolysed from Connaught Medical Research Laboratories, University of Toronto, Canada; batch 346-1 at 5% in gel buffer) in a continuous Tris (0.167M) - EDTA (disodium salt 0.005M) - boric acid (0.024M) tank buffer at pH 8.6. The gel buffer was a 30% aqueous solution of the tank buffer. A constant voltage of 12 volts/cm was applied for ninety minutes. The faster migrating haemoglobin was recorded as A, the slower as B, no other variants were seen.

Transferrin type:- This was determined by starch gel electrophoresis (12½% solution, batch 329-1 from Connaught Medical Research Laboratories). The buffer system was discontinuous. Tris-hydrochloric acid was used as a gel buffer (0.008M Tris was adjusted to pH 8.0 with hydrochloric acid). Boric acid (0.3M) - sodium hydroxide (0.1M) at pH 8.7 was used as a tank buffer. A constant voltage of 12 volts/cm was applied for 200 minutes. Gels were stained overnight with nigrosin stain (BDH, England; 1g. dry nigrosin powder/litre of 7.4% glacial acetic acid, 55.6% methanol and 37% distilled water). Gels were then fixed with a 50/50/10 mixture of methanol distilled water and acetic acid. Five alleles were detected. They were types/

types A, B, C, D and E as described by Cooper et al. (1967). Transferrins now labelled G and M may have been present but were undetected.

R-O-i Type:- A standard haemolytic test was used. The red cell suspensions were obtained from ACD-blood (7 ml. Becton-Dickinson ACD vacutainers were used). Red blood cells were washed three times in 0.9% saline. A 0.5% red blood cell suspension was used for testing. Antisera to R and O blood group antigens were kindly supplied by Dr. Tucker. Both antisera were diluted 1/4 in 0.9% saline before use. Pooled rabbit serum was used as a complement source. Cross-absorptions with heat-inactivated rabbit serum using red blood cells from twenty sheep revealed the presence of two antibodies. One antibody reacted with twenty out of twenty sheep. This may be a species specific antibody. The other antibody only reacted with R positive sheep (eight positive and twelve negative). Consequently, all complement was absorbed with red blood cells from two R-positive sheep before use. Absorptions were made using a 1:1 mixture of red blood cells to complement. Absorptions were carried out for thirty minutes at $4^{o}C$.

In the test, 25 ul. of diluted antiserum was added to 25 ul. of 0.5% red blood cell suspension. After fifteen minutes 25 ul. of absorbed rabbit serum was added as a complement source. 0.9% saline was used as a negative control. Bacto-Sheep Haemolysin Glycerinated (DIFCO, Michigan, U.S.A.) diluted 1/512 in 0.9% saline was used as a positive control.

The ii genotype was inferred from the absence of types R and O on the sheep erythrocytes. R positive sheep are genetically R-I-, while O positive sheep are $r^{o}r^{o}I$-. (Rasmusen, 1962).

5. Methods of Analysis

Sera which appeared similar when examined by eye were grouped together to form clusters. No serum was assigned to more than one cluster, although there were strong indications that several sera contained more than one specificity. This assignment was subsequently confirmed by chi-squared analysis. The computer programme used is given in the appendix. All sera were used in antigen definition. No serum was unassigned. Within each cluster one of two procedures was followed to produce the serographs. In clusters with few sera, the sera were sorted individually, (i.e. all the animals reacting with one serum were listed first, this was followed by all the animals which reacted with the second but not the first serum, this in turn was followed by all the animals which reacted with the third, but not the first or the second serum and so on. Within the reactions of the first serum, animals which reacted with the second serum were given precedence over animals which reacted with the third serum. Also, animals which reacted with the first and the third sera were given precedence over those which only reacted with the first and fourth sera and so on). In clusters with several sera a different procedure was followed. The sum of the reactions for each serum was added to produce a total for each animal. Serum reactions were scored '4', '3', '2', '1' or '0'. A negative reaction (less than 40% killed lymphocytes) was given a '0'. Unreadable reactions were given a '1'. A reaction giving 40-60% lymphocyte killing was scored as a '2', 61-94% killing was scored as a '3', 95-100% killing was scored as a '4'. Subsequently, I refer to strong positives and weak positives (partials). A '4' is a strong positive/

positive, '2' and '3' are weak positives. Animals were then arranged by their totals. Animals with the highest scores went to the top, animals with low scores went to the bottom. In the serographs zeros have been left blank. Each cluster was then examined to see if any antigens could be detected by the groups of sera.

Results

1. Accuracy of Antigen Assignment

Evidence for the accuracy and reliability of the procedure used to assign antigens to sheep comes from three sources.

a) The reproducibility of the cytotoxic test:- In this series of tests, five animals were tested on two different occasions. There were thirty-eight discrepancies in 597 repeated tests. The estimate for reproducibility is 93.6%. This is a minimum estimate. The five animals were tested twice because the cell preparations were initially of poor quality. Both the initial tests and the repeat tests have been included in the analysis.

b) Repeatibility of antigen assignment:- The five animals which were tested on two different occasions also showed concordant antigen assignment. In the initial tests a total of eleven specificities were assigned. In the repeat tests, there were no discrepancies.

c) Specificities detected by different antisera from the same sheep:- Fifteen different sheep each provided two serum samples. In fourteen of the fifteen pairs, both sera were assigned to the same specificity. The exceptional pair or sera came from a sheep which lambed in the intervening interval. This sheep produced antibodies against its foetus. As sera were assigned without regard to their origin, the result suggests that sera detecting the same specificity were assigned to the same cluster.

Further confirmation that specificities are being assigned correctly comes from the observation that the defined specificities are inherited in families.

2. Specificities Present

Analysis of the data suggested the existence of several lymphocyte antigens. I have discussed the sera defining each proposed specificity separately. For each specificity, the origin of the sera is given in Table 2, a serograph is given in Table 3 and the correlation coefficients between sera are given in Table 4. The inheritance of each specificity is given in Table 5.

As the specificities have been defined, it is not necessary for an animal to react with all sera in a cluster. Several sera apparently fail to react with an animal although the animal's lymphocytes appear to possess the relevant antigen. For some sera this may be due to their being used near the end-point. Other sera only miss/

miss the occasional reaction with positive sheep. This is probably because the reproducibility of the cytotoxic test used is less than 100%. The irreproducibility will take two forms, spurious extra reactions (false positives) and missed reactions (false negatives). The effect of false positives is dependent upon the number of sera in the cluster. This point has been dealt with in the introduction to this chapter. False negatives can be partly allowed for by not insisting that positive animals must react with all sera. Even a low frequency of false positives will have an appreciable effect. For example, if a cluster is composed of twenty sera and if the frequency of false positives is 3%, by assuming that errors are randomly distributed, the expected distribution of missed reactions can be calculated from the binomial expansion. Thus, only 54.3% of antigen positive sheep are expected to react with all twenty sera. 33.6% of positive sheep are expected to react with nineteen sera, 9.9% with eighteen sera, 1.8% with seventeen sera and a mere 0.2% with sixteen sera. Only 0.2% of positive sheep are expected to react with fifteen or less sera. The presence of low-titre antisera means that the binomial expansion cannot be rigourously applied to all clusters. However, the general points are clear. Antigen definition must make allowance for missed reactions. I have tried to allow for missed reactions in defining specificities.

Another interesting consequence of irreproducibility is that even identical and monospecific antisera are not always expected to give identical results. However, correlation coefficients of 1.0 are quite common in the literature. It may be that monospecific antisera give much better reproducibility than less specific antisera.

In the genetic studies, several antigen-positive lambs appeared to come from antigen-negative parents. This could have one of three explanations. Firstly, there could be a complex genetic mechanism involved in the inheritance of the antigen. I believe that this is unlikely. The remaining families showed the inheritance expected from an autosomal dominant gene. Secondly, there could be an error in antigen determination. Thirdly, there could have been a parentage error. Data presented in Chapter 6 strongly favours a parentage error. Each animal involved in anomalous antigen inheritance also showed unusual inheritance for at least one other lymphocyte antigen. The inheritance of haemoglobins, potassium type, transferrins and R-O-i blood types showed no discrepancies. However, in many families, both parents were homozygous for the same alleles and false parentages would not be detected.

With a dominant gene, the expected inheritance will be different if the parent is homozygous or heterozygous. A homozygous parent will transmit the specificity to all his offspring regardless of the genotype of the other parent. A heterozygous parent will transmit the specificity to only half his offspring. With a dominant gene, one cannot distinguish between homozygotes and heterozygotes. However, if Hardy-Weinberg is assumed, when a gene is rare the frequency of heterozygotes will greatly outnumber the frequency of homozygotes. Therefore, one expects the majority of families studied, with only one positive parent, to show approximately 1:1 segregation of positive to negative offspring. If the gene is recessive, when only one parent is positive, all offspring should be negative. The terms dominant and recessive are used to compare presence versus absence.

Until the specificities can be confirmed by absorptions and further family studies, I have given them provisional designations. The specificities are numbered from 1 onwards (i.e. 1, 2, 3, etc.). They are given the designation 'P' to denote their provisional status. The letter 'S' before 'P' refers to the fact that these are sheep antigens.

The sera used to define each cluster are given in Table 2.

SP 1

This specificity is detected by nine sera. The nine are listed in Table 2.1. A serograph presenting the reactions of the nine sera is given in Table 3.1. In the serograph, the serum designations are given in the top three rows (021, 071, 096, etc.). The animal reactions are given on the left hand side. Thus, animal 093 gave '4' reactions against all nine sera. Animal 241 only reacted against serum 919. I have called six animals positive and 252 negative for specificity SP 1. The six positive sheep are animals 93, 33, 110, 116, 237 and 90. Five of the sera gave identical reactions. The similarity of the sera is unlikely to be due to chance. All comparisons between sera gave a probability of $<10^{-6}$, by Fisher Exact Test, that the reactions were due to chance.

Two sera each gave one additional reaction. Serum 072 was a repeat bleed from the same donor that provided serum 071. Serum 072 was taken one month after serum 071 and was not immunised between the two bleeds. The extra reactions could be due to a non-specific reaction or to a contaminating antibody in the serum. The irreproducibility/

Table 2

Key:		
	SB	Scottish Blackface
	FD	Finnish Landrace x Dorset Horn
	WM	Welsh Mountain
	FL	Finnish Landrace
	D	Damline
	GML	German Merino Landrace
	C	Cheviot
	CF	Clun Forest
	RM	Romney Marsh
	BL	Border Leicester
	O	Oxford Down
	SD	Southdown
	SCBL	Suffolk x Cheviot x Border Leicester
	P	Prealpe

Table 2.1

Sera Defining Specificity SP 1

Serum Identity	Breed of Origin	Country of Origin	Laboratory Designation	Immune (I) or Parous (P)	Test Dilution
021	SB	Scotland	1N180	P	1/8
071	SB	Scotland	2N223	P	1/16
096	SB	Scotland	2T278	P	1/16
125	FD	Scotland	64363 20.2.78	P	1/2
142	FD	Scotland	64620	P	N
919	WM	England	80/1W	I	N
072	SB	Scotland	2N223 22.8.77	P	1/4
028	SB	Scotland	1T122	P	1/8
903	P	France	Anti - 3	I	1/8

TABLE 3.1

SEROGRAPH OF SPECIFICITY SP1

```
      000119009
      279241720
      116529283

033 444444444
093 444444444
110 444444444
116 444444444
237 444444444
098 444434444
------------
241       4
109        4
045         4
242         4
246         4
247         4
248         4
249         4
114          4
130          4
172          4
182          4
191          3
083          3
098          3
      000119009
      279241720
      116529283
```

Table 4.1

SP 1

Number	Serum									
1.	021	-								
2.	071	1.0	-							
3.	096	1.0	1.0	-						
4.	125	1.0	1.0	1.0	-					
5.	142	1.0	1.0	1.0	1.0	-				
6.	919	N/A	N/A	N/A	N/A	N/A	-			
7.	072	N/A	N/A	N/A	N/A	N/A	842	-		
8.	028	N/A	N/A	N/A	N/A	N/A	628	628	-	
9.	903	N/A	N/A	N/A	N/A	N/A	600	600	442	-
		0	0	0	1	1	9	0	0	9
		2	7	9	2	4	1	7	2	0
		1	1	6	5	2	9	2	8	3
		1.	2.	3.	4.	5.	6.	7.	8.	9.

Table 4

Correlation Coefficients between the Sera Within each Cluster

Correlation coefficients have not been presented for specificities SP 11, SP 13, SP 14 and SP 15 as these specificities were each defined by a single serum. All correlation coefficients have been multiplied by X1000 for presentation purposes.

Key:- N/A The comparison could not be carried out as one serum was completely contained within the other. The nature of the inclusion can be obtained from the serographs.

* The comparison between the two sera gave a negative correlation coefficient (i.e. $bc > ad$ where a, b, c and d are the terms in a 2 x 2 table.

irreproducibility could be accounted for by assuming that the contaminating antibody was not being tested at the optimal titre. The remaining two sera both had definite tails.

The SP 1 specificity is comparatively rare. There are only six positives in 258 animals tested. Four of the positive animals came from three families (Table 5.1). In these three families the mothers were positive and the fathers negative. One mother (animal 90) had three offspring. One of these was positive (animal 93) and two were negative. I was able to test a total of three offspring from the other two mothers. All three were negative. The absence of the specificity in female offspring could suggest an X-linked recessive gene. If this were the case, a negative male (y-) crossed to a positive female (++) should only produce positive males (y+) and negative females (-+). As some male offspring are negative this excludes this mode of inheritance. One lamb positive out of seven is consistent with a 1:1 segregation of a dominant gene (P = 0.062 from the binomial expansion).

As the genetic evidence favours a dominant mode of inheritance, I shall assume that this specificity is controlled by a dominant gene.

The breeds of the positive animals and the breed of origin of the sera are also of interest. Animals 90, 93, 110 and 116 were Damline sheep from Skedsbush farm in Scotland. Animal 33 was a Dorset Horn ram from the experimental farm of the Animal Diseases Research Organisation Farm at Moredun, Edinburgh. Animal 237 was a Welsh Mountain ewe imported from Wales. This implies that the same specificity is present in the Damline, Dorset and Welsh Mountain breeds.

Table 5.1

Inheritance of Specificity SP 1

Parents		Offspring		
Phenotype R E	Number R E	Phenotypes M	F	Totals (M+F)
		+ : -	+ : -	+ : -
+ x +	0 0	- -	- -	- -
+ x -	0 0	- -	- -	- -
- x +	2 3	1 2	0 3	1 5
- x -	11 55	0 38	0 53	0 91
TOTALS	11 58	1 40	0 56	1 96

Key: R Ram
E Ewe
M Male Offspring
F Female Offspring

N.B. Rams were sometimes used on more than one category of ewe.

Similarly, with the breed of origin of the sera, five sera came from Scottish Blackface, two from a Finnish Landrace x Dorset Horn cross, one from Welsh Mountain on an English farm and one from a French Prealpe. This implies that the specificity is present in these breeds also. As mentioned in the introduction, immunisations were carried within and not between breeds.

The SP 1 specificity is present in the Dorset Horn, Damline and Finnish Landrace x Dorset Horn flocks. This observation is perhaps not too surprising. Both the latter lines were developed from Dorset Horn crosses.

SP 2

Twenty-two sera gave similar reactions and I have grouped these together to form cluster SP 2 (Table 3.2). Two sera 206 and 218 were repeat bleeds from the same animal. Serum 218 was taken four months after serum 206. The sources of the sera are given in Table 2.2. To aid interpretation of the serograph, I have added the row totals at the side of each row. A graph of the number of animals reacting with a given number of antisera versus the number of positive sera is presented in Figure 1. This shows the typical U-shaped distribution expected from a group of multi-specific sera with a shared specificity (Bodmer et al., 1969). On the basis of this graph, it appears to me that animals reacting with sixteen or more sera probably possess a specificity common to the twenty-two antisera. Twenty-five sheep were not included in this histogram as they were not tested against all twenty-two antisera. Animals

that/

Table 2.2

Sera Defining Specificity Cluster SP 2

Serum Identity	Breed of Origin	Country of Origin	Laboratory Designation	Immune (I) or Parous (P)	Test Dilution
214	FL	Scotland	4E40 4.12.77	P	1/2
206	FL	Scotland	1732 10.8.77	P	1/8
005	SB	Scotland	1N050	P	1/2
218	FL	Scotland	1732 4.12.77	P	1/4
159	FD	Scotland	7G341 13.2.78	I	1/8
171	D	Scotland	7J456 5.5.78	P	1/8
122	FD	Scotland	64560 20.2.78	P	1/2
161	FD	Scotland	7G391 20.2.78	I	1/8
148	FD	Scotland	64489 15.3.78	P	1/2
123	FD	Scotland	64392 20.2.78	P	1/4
198	WM	Scotland	6W12 18.7.78	P	N
914	GML	W. Germany	9111	P	N
194	C	Scotland	95C0Q 20.7.78	P	N
912	GML	W. Germany	9110	I	1/4
166	FD	Scotland	7G413 27.2.78	I	1/8
167	FD	Scotland	7G418 27.2.78	I	1/2
132	FD	Scotland	64526 27.2.78	P	N
922	CF	England	66/0	P	N
172	D	Scotland	7J063 11.5.78	P	1/2
154	FD	Scotland	64368 20.2.78	P	1/2
097	SB	Scotland	2T277	P	1/4
920	RM	England	188/2	I	N

TABLE 3.2

SEROGRAPH OF SPECIFICITY SP2

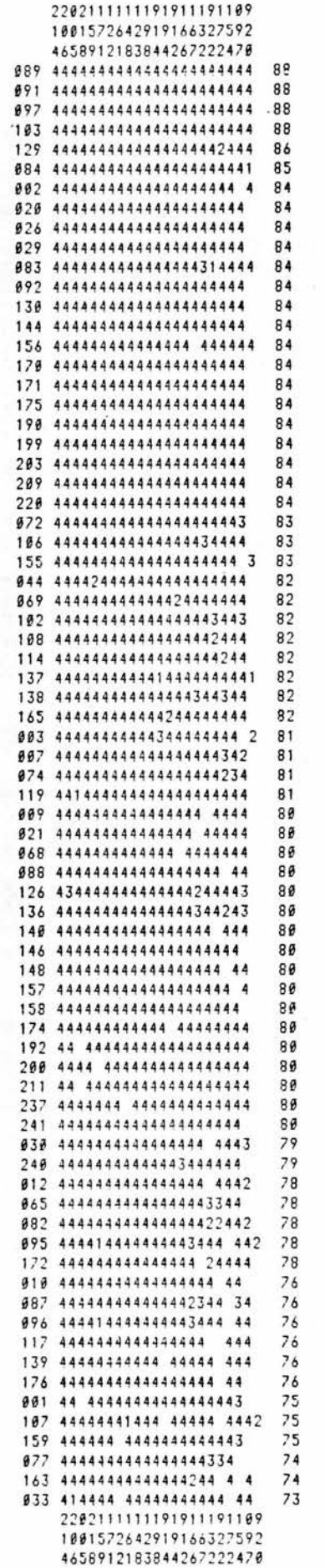
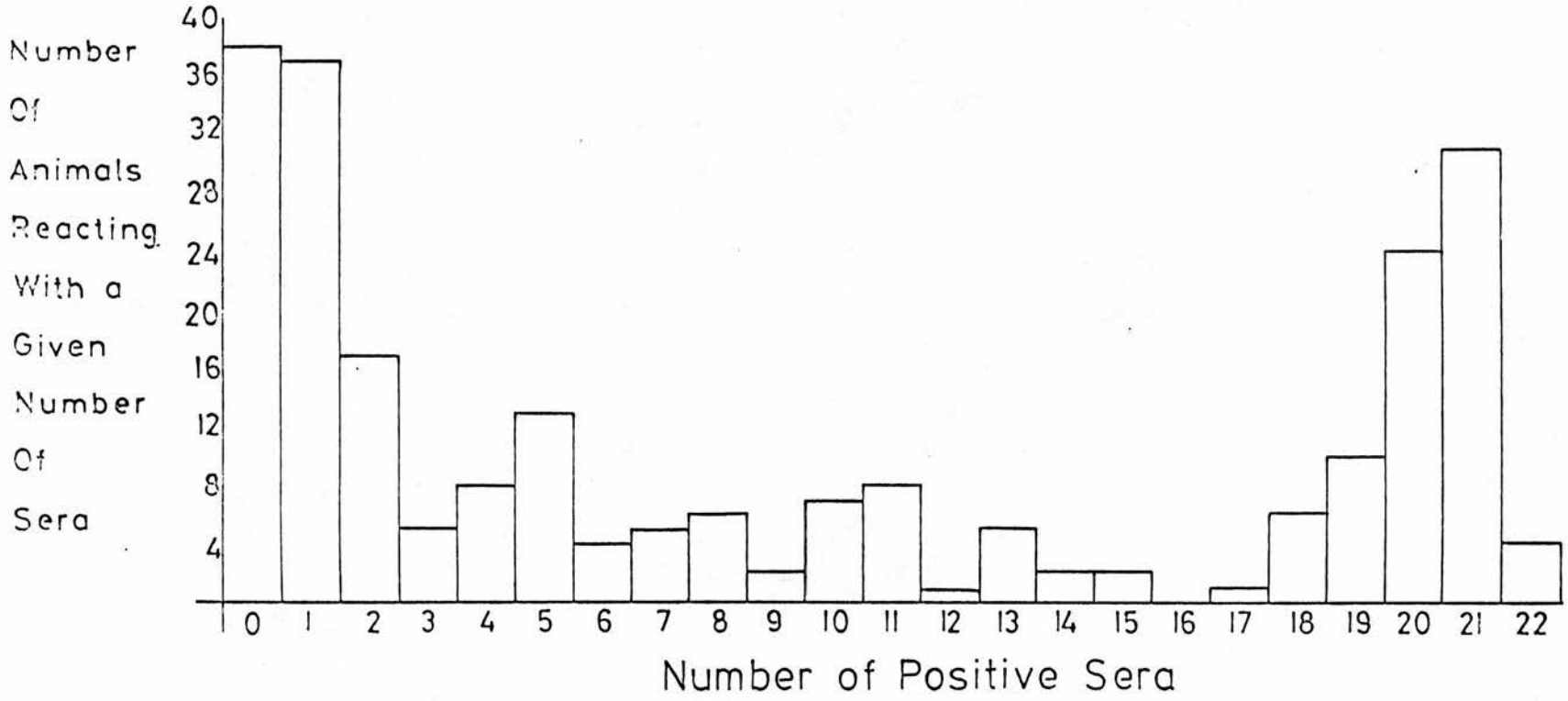


Table 4.2

SP 2																							
1.	214	-																					
2.	206	770	-																				
3.	005	546	553	-																			
4.	218	747	N/A	612	-																		
5.	159	703	697	579	688	-																	
6.	171	689	671	590	674	687	-																
7.	122	576	591	541	598	596	591	-															
8.	161	670	651	569	653	636	596	554	-														
9.	148	515	513	482	545	541	534	595	358	-													
10.	123	590	599	544	600	611	606	692	429	609	-												
11.	198	599	629	493	607	655	604	685	416	705	703	-											
12.	914	643	624	509	634	562	666	702	363	686	657	590	-										
13.	194	609	608	712	640	603	673	635	447	614	613	408	619	-									
14.	912	562	576	565	613	550	624	697	390	671	655	509	679	772	-								
15.	166	714	735	560	700	744	672	602	508	594	613	509	458	484	452	-							
16.	167	698	733	560	685	744	658	589	497	570	600	512	432	488	441	741	-						
17.	132	513	533	396	534	532	479	564	314	611	539	525	495	369	419	538	521	-					
18.	922	520	501	356	506	438	438	529	318	545	516	499	575	343	401	443	437	591	-				
19.	172	401	439	406	423	425	441	371	334	348	346	230	349	518	509	450	433	288	207	-			
20.	154	465	486	420	508	501	476	564	319	598	519	487	501	394	440	531	481	581	525	316	-		
21.	097	271	341	410	357	301	369	409	263	499	440	353	415	466	543	348	294	382	344	435	459	-	
22.	920	332	282	247	260	210	242	186	167	155	191	170	178	194	190	237	211	182	181	180	162	076	-
		2	2	0	2	1	1	1	1	1	1	1	9	1	9	1	1	1	9	1	1	0	9
		1	0	0	1	5	7	2	6	4	2	9	1	9	1	6	6	3	2	7	5	9	2
		4	6	5	8	9	1	2	1	8	3	8	4	4	2	6	7	2	2	2	4	7	0
		1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.	13.	14.	15.	16.	17.	18.	19.	20.	21.	22.

Figure 1

Reactions of 233 Sheep Against 22 Sera of the SP 2 Cluster.



that reacted with sixteen or more of the sera in this cluster, I have called positive for SP2.

The inheritance of the SP 2 specificity is presented in Table 5.2. Two positive lambs came from a cross between two negative parents. The two lambs were littermates. There are several explanations for the anomalous antigen inheritance. Firstly, there may be a complex genetic mechanism involved in the inheritance of the antigen. I believe that this is unlikely. The remaining families show the segregation expected from a trait determined by an autosomal dominant gene. These other families also rule out a recessive gene. Secondly, there could be an error in detecting the specificities of the offspring or the specificities of their parents. I believe that this too is not the case. The lambs (animals 91 and 92) react with 21/22 and 22/22 sera. This could be due to the lambs reacting with the tails of the twenty-two sera but it is not likely. The father of animal 025 reacts with only 4/22 sera. He was also mated to five other negative ewes. These matings produced a total of nine lambs. All nine lambs were negative. This suggests that the father does not possess the SP 2 specificity. The mother, animal 090, reacted with 7/22 sera. There were three offspring, the third offspring (animal 093) reacted with six of the seven sera that the mother was positive for. The test with the other serum for which the mother was positive, was 'unreadable'. The mother could be positive for SP 2. The concordance with the third offspring over all the sera tested makes this unlikely. The final explanation is false parentage. This explanation would appear most likely./

Table 5.2

Inheritance of Specificity SP 2

Parents		Offspring					
Phenotype R E	Number R E	Phenotypes M		F		Totals (M+F)	
		+ : -		+ : -		+ : -	
+ x +	3 8	4	2	7	2	11	4
+ x -	3 14	4	4	9	6	13	10
- x +	4 11	4	5	8	5	12	10
- x -	7 25	1	17	1	18	2	35
TOTALS	11 58	13	28	25	31	38	59

likely. Although haemoglobin, red cell potassium and R-O-i determinations did not confirm this.

If we ignore these two lambs, the other pedigrees suggest an autosomal dominant mode of inheritance for SP 2. Sera from a variety of breeds detect the SP 2 specificity. These include the Finnish Landrace, Welsh Mountain, Cheviot, Scottish Blackface, Clun Forest, German Landrace, Damline and Finnish Landrace x Dorset Horn crosses. In my sample, animals from several breeds possessed the specificity. Positive breeds included Finnish Landrace, Damline, Welsh, Dorset Horn, Texel and Wensleydale. This suggests the specificity is present in several different breeds. There is a relatively high frequency of positive animals in my sample (85/258). Further, 22/120 sera from a variety of sources carried this specificity as the major antibody. These two observations both suggest that SP 2 is a fairly common specificity.

SP 3

There are fourteen sera in this cluster (Table 2.3). Serum 042 was tested at two different titres. Serum 042A was tested undiluted, serum 042B was tested at a dilution of 1/4. Sera 041, 042A and 042B were from the same sheep. Sera 041 and 042 were taken one month apart. There was no known intervening immunising stimulus. Sera 067 and 068 also came from the same donor. Serum 068 was taken fifteen months after serum 067. The donor was mated in the intervening interval but the mating proved to be barren. Sera 093 and 094 also came from the same donor. They were taken one year apart.

The animal was mated in the interval. The mating produced one lamb. There was no increase in titre or reaction frequency in the second sample. The fourteen sera have been arranged in a serograph in Table 3.3. As before, the reactions for each animal have been summed and the totals placed beside each row.

The sera appear to form three distinct groups. Group A contains the first six sera (042A, 042B, 041, 046, 093 and 203). Group B contains the seven sera (909, 239, 094, 170, 190, 067 and 068). Serum 111 forms group C. The simplest interpretation is that the group A sera detect two antigens. The group B sera detect one of the two antigens while the group C serum detects the other.

As a working hypothesis, I shall assume that groups B and C each detect a single specificity. The two specificities may or may not be genetically related. I have called the specificities detected by the two groups SP3A and SP3B. Separate serographs for each are presented in Tables 3.3A and 3.3B. The SP3A cluster is defined by six sera which were specific for both SP3A and SP3B and by one serum (111) which was specific for SP3A alone. Eight animals which were SP3B positive failed to react with serum 111. As their reactions were probably due to their SP3B specificity, they were scored as SP3A negative. They have been indicated in the serograph by a dot on the left hand side of the animal number. I have called all remaining animals which reacted with three or more sera positive. The inheritance of the SP3A specificity is given in Table 5.3A. With the exception of three positive males and two positive females arising from -x- matings, the family studies are in agreement with an autosomal dominant mode of inheritance. The five positive offspring came from three matings. As before, I suspect that the parents of these animals may have been wrongly assigned. Of course other explanations involving false antigen assignment or complex genetic control of the detected antigen cannot be excluded.

Table 2.3

Sera Defining Specificity SP 3

Serum Identity	Breed of Origin	Country of Origin	Laboratory Designation	Immune (I) or Parous (P)	Test Dilution
042A	SB	Scotland	1T393 22.8.77	P	N
042B	SB	Scotland	1T393 22.8.77	P	1/4
041	SB	Scotland	1T393	P	1/4
046	SB	Scotland	1T465	P	1/8
093	SB	Scotland	2T238	P	1/4
203	FD	Scotland	2E11 10.8.77	P	1/2
909	GML	W. Germany	9752	I	1/8
239	BL	Scotland	5L22 9.2.78	I	1/8
094	SB	Scotland	2T238 25.7.78	P	1/2
170	D	Scotland	7J450 3.4.78	P	1/8
190	BL	Scotland	BL8 9.10.77	P	N
067	SB	Scotland	2N215	P	1/8
068	SB	Scotland	2N215 12.10.78	P	1/4
111	SB	Scotland	2T095 25.7.78	P	1/2

TABLE 3.3

SEROGRAPH OF SPECIFICITY SP3

	00000292011001 44449003979661 22163399400781 AB	
045	444444444444	56
036	4444444444444	52
043	4444444444444	52
085	4444444444444	52
098	44 44444444444	52
109	4444444444423 4	49
035	444444 444444	48
083	444 444444444	48
100	44 4444444444	48
152	4444444444 44	48
241	44414444444 4	45
150	444444444 4 4	44
175	4444444444 4	44
004	4444444 44 4	40
166	44444444 4 4	40
116	444444 4434	39
147	444 443244 4	37
167	444244 443 4	37
125	111444244 4114	35
132	44444424 1 4	35
075	444444 24 4	34
124	4444244 4 4	34
142	444444 24 4	34
087	444444 4 4	32
169	4444444 4	32
170	444444 4 4	32
097	444444 3 4	31
128	444444 2 4	30
141	444444 2 4	30
095	444444 4	28
117	4444444	28
133	4444242 4	28
134	4444 44 4	28
138	444444 4	28
025	4444 43 4	27
123	444444 3	27
118	144444 1 4	26
127	444441 4	25
005	44 4 44 4	24
028	4444 4 4	24
081	444444	24
086	444444	24
094	4442442	24
101	4 444 4 4	24
135	44444 4	24
140	4444 4 4	24
151	4444 4 4	24
172	4444 4 4	24
174	444444	24
088	44144 3 3	23
148	44 442 4	22
066	44444	20
074	44444	20
153	4444 4	20
173	444 4 4	20
060	4442 4	18
163	414 44	17
108	444 4	16
073	2 4 3 4	13
155	4 44	12
240	2 2 44	12
	00000292011001 44449003979661 22163399400781 AB	

	00000292011001 44449003979661 22163399400781 AB	
031	4 1 4	9
024	4 4	8
032	4 4	8
037	4 4	8
072	4 4	8
099	4 4	8
104	4 4	8
131	4 4	8
159	4 4	8
162	4 4	8
187	44	8
029	43	7
080	3 4	7
022	2 4	6
065	2 4	6
067	2 4	6
079	2 4	6
115	2 4	6
126	11 4	6
149	24	6
176	2 4	6
248	2 4	6
027	1 4	5
091	1 4	5
122	4 1	5
252	1 4	5
006	4	4
014	4	4
015	4	4
051	4	4
059	4	4
064	4	4
071	4	4
076	4	4
078	4	4
082	4	4
092	4	4
145	4	4
154	4	4
158	4	4
160	4	4
161	4	4
177	4	4
178	4	4
182	4	4
183	4	4
188	4	4
189	4	4
203	4	4
233	4	4
234	4	4
242	4	4
244	4	4
246	4	4
249	4	4
250	4	4
251	4	4
254	4	4
256	4	4
110	3	3
239	3	3
	00000292011001 44449003979661 22163399400781 AB	

Table 4.3

SP 3

1.	042A	-												
2.	042B	811	-											
3.	041	779	836	-										
4.	046	808	822	796	-									
5.	093	522	534	504	512	-								
6.	203	695	748	714	726	510	-							
7.	909	560	555	508	506	397	569	-						
8.	239	501	476	423	448	N/A	436	541	-					
9.	094	N/A	N/A	482	509	N/A	481	542	733	-				
10.	170	520	489	401	425	N/A	427	489	682	683	-			
11.	190	461	N/A	405	427	N/A	N/A	481	644	645	516	-		
12.	067	372	349	264	333	295	302	476	608	594	608	647	-	
13.	068	323	301	233	267	394	260	431	600	545	501	604	664	-
14.	111	276	268	255	283	288	225	122	095	116	136	010	021	001

	0	0	0	0	0	2	9	2	0	1	1	0	0	1
	4	4	4	4	9	0	0	3	9	7	9	6	6	1
	2	2	1	6	3	3	9	9	4	0	0	7	8	1
	A	B												
	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.	13.	14.

TABLE 3.3A

SEROGRAPH OF SPECIFICITY SP3A

```
      0000021
      4444901
      2216331
      AB
 004 4444444  28
 045 4444444  28
 075 4444444  28
 087 4444444  28
 095 4444444  28
 097 4444444  28
 109 4444444  28
 128 4444444  28
 132 4444444  28
 138 4444444  28
 141 4444444  28
 166 4444444  28
 169 4444444  28
 170 4444444  28
 175 4444444  28
 123 4444443  27
 124 4444244  26
 133 4444244  26
 167 4442444  26
 118 1444444  25
 127 4444414  25
 025 4444 44  24
 028 4444 44  24
.035 444444   24
.036 444444   24
.043 444444   24
 081 444444   24
.085 444444   24
 086 444444   24
 098 44 4444  24
 116 444444   24
 117 444444   24
 134 4444 44  24
 135 44444 4  24
 140 4444 44  24
 142 444444   24
 147 444 444  24
.150 444444   24
 151 4444 44  24
.152 444444   24
 174 444444   24
 094 444244   22
 241 444144   21
 066  44444   20
 074  44444   20
.083 444 44   20
 088  441443  20
.100 44 444   20
 101 4  4444  20
 153 4444  4  20
 172 4444  4  20
 173  444 44  20
 125 1114444  19
 060 4442 4   18
 163 414 44   17
 005 44  4 4  16
 108 444   4  16
 148  44 44   16
 ---------------
 024     4 4   8
 031      44   8

      0000021
      4444901
      2216331
      AB
```

```
      0000021
      4444901
      2216331
      AB
 032      44   8
 072     4 4   8
 099     4 4   8
 104     4 4   8
 131     4 4   8
 155     4 4   8
 159     4 4   8
 162 4   4     8
 080     3 4   7
 022     2 4   6
 065     2 4   6
 073     2 4   6
 079     2 4   6
 115     2 4   6
 126   11  4   6
 176     2 4   6
 240     2 4   6
 248      24   6
 091     1 4   5
 252 1   4     5
 006       4   4
 014       4   4
 015       4   4
 027       4   4
 029      4    4
 037     4     4
 059      4    4
 064       4   4
 071       4   4
 076       4   4
 078       4   4
 082       4   4
 092       4   4
 122 4         4
 145       4   4
 149       4   4
 154       4   4
 158     4     4
 160       4   4
 161       4   4
 177       4   4
 178       4   4
 182     4     4
 183     4     4
 187      4    4
 188       4   4
 189     4     4
 203     4     4
 233       4   4
 234       4   4
 242       4   4
 244       4   4
 246       4   4
 249       4   4
 250       4   4
 251       4   4
 254       4   4
 256       4   4
 110     3     3
 239      3    3
 067     2     2
 202     2     2
      0000021
      4444901
      2216331
      AB
```

Table 5.3A

Inheritance of Specificity SP 3A

Parents		Offspring					
Phenotype R E	Number R E	Phenotypes M		F		Totals (M+F)	
		+	-	+	-	+	-
+ x +	1 3	2	1	3	1	5	2
+ x -	1 5	0	3	4	4	4	7
- x +	5 15	5	2	7	9	12	11
- x -	10 35	3	25	2	26	5	51
TOTALS	11 58	10	31	16	40	26	71

The serograph of the seven remaining sera which form the SP3B cluster is presented in Table 3.3B. I have taken a reaction with five or more sera to mean that the animals possess the antigen. Only twelve cells reacted with five or more of the seven sera. Cells 35 and 36 were repeats from the same animal. Animal 45 was the offspring of animal 43. Unfortunately, the father could not be typed. Consequently, this pair have not been included in the family studies. One positive male (animal 109) came from a -x- mating (Table 5.3B). With the exception of this animal, the specificity appears to be inherited as an autosomal dominant. Animal 109 is the exception. This animal also showed an unusual inheritance of the SP3A specificity. If SP3A and SP3B are coded for by different genes, then two observations of unexpected inheritance support the explanation of false parentage.

The SP3B specificity is present in the Border Leicester and Damline breeds. The sera came from Scottish Blackface, Damline and Border Leicester animals.

SP 4

The fourth cluster involves ten sera. The serograph of the ten sera is presented in Table 3.4. The source of the ten sera is given in Table 2.4. Sera 087 and 114 were different bleeds from the same donor. One serum (129) was tested both at neat (129A) and at a dilution of 1/8 (129B). I believe two different specificities are detected by this cluster of sera. Sera 917 and 087 detect the first specificity./

TABLE 3.3B

SEROGRAPH OF SPECIFICITY SP3B

```
     2011009
     3979660
     9400789

036 4444444  28
043 4444444  28
045 4444444  28
083 4444444  28
085 4444444  28
098 4444444  28
100 4444444  28
035 444444   24
152 444 444  24
241 4444 44  24
109 44423 4  21
150 44 4 44  20
--------------
125 44 4112  16
175 444   4  16
116 4434     15
147 244   3  13
004  44   4  12
166 4 4   4  12
167 443      11
142 24 4     10
005  44       8
124  4    4   8
073 4 3      7
132 4   1 2  7
075 24       6
148    4 2   6
240 2    4   6
037 4        4
051     4    4
067      4   4
087    4     4
101   4      4
117       4  4
134       4  4
155      4   4
169       4  4
170  4       4
172       4  4
187       4  4
025       3  3
029       3  3
088   3      3
097  3       3
094       2  2
128    2     2
133       2  2
141   2      2
149      2   2
     2011009
     3979660
     9400789
```

Table 5.3B

Inheritance of Specificity SP3B

Parents				Offspring					
Phenotype R	E	Number R	E	Phenotypes M		F		Totals (M+F)	
				+	-	+	-	+	-
+ x +		0	0	-	-	-	-	-	-
+ x -		0	0	-	-	-	-	-	-
- x +		2	3	2	0	1	4	3	4
- x -		11	55	1	38	0	51	1	89
TOTALS		11	58	3	38	1	55	4	93

Table 2.4

Sera Defining Specificity SP 4

Serum Identity	Breed of Origin	Country of Origin	Laboratory Designation	Immune (I) or Parous (P)	Test Dilution
917	CF	England	151/0	P	N
087	SB	Scotland	2T156	P	1/2
088	SB	Scotland	2T154	P	1/2
106	SB	Scotland	2T403	P	1/2
114	SB	Scotland	2T156 25.7.78	P	N
129A	FD	Scotland	64466 27.2.78	P	N
129B	FD	Scotland	64466 27.2.78	P	1/8
905	GML	W. Germany	9755	I	1/8
924	FL	Scotland	5L2093 16.11.77	P	1/2
178	FL	Scotland	5L2093 12.12.77	I	3/8

TABLE 3.4

SEROGRAPH OF SPECIFICITY SP4

	9001111991 1880122027 7786499548 AB	
062	4444444444	40
024	4 44444444	36
063	444444444	36
099	4 44444444	36
075	44444444	32
100	44444444	32
110	44444444	32
111	44444444	32
112	44444444	32
120	44444444	32
093	144 44444	29
107	41444444	29
025	4444444	28
071	44 44444	28
090	44444 44	28
142	4444444	28
143	4444444	28
145	4444444	28
146	4444444	28
154	4444444	28
161	4444444	28
052	444 444	24
078	44 4444	24
144	444444	24
152	444444	24
156	444444	24
168	44444 4	24
042	44444 4	24
043	444444	24
227	44444 4	24
252	44444 4	24
038	44444 2	22
228	444442	22
006	4 4 44 4	20
039	44444	20
040	44444	20
041	44444	20
044	44444	20
066	44444	20
129	44444	20
136	44444	20
182	44444	20
183	44444	20
189	44444	20
202	44444	20
225	44444	20
230	44444	20
231	44444	20
246	4444 4	20
229	4 4424	18
247	42 4 44	18
201	44144	17
203	4 444	16
239	44 4 4	16
245	4444	16
253	4 444	16
254	44 4 4	16
	9001111991 1880122027 7786499548 AB	

	9001111991 1880122027 7786499548 AB	
256	4 4 3 4	15
037	4 442	14
060	2444	14
106	44 42	14
031	4 4 4	12
109	4 4 4	12
160	4 4 4	12
217	4 44	12
243	44 4	12
238	3 44	11
257	44 3	11
068	24 4	10
244	4 2 4	10
249	44 2	10
091	1 4 4	9
233	4 41	9
023	44	8
061	44	8
064	42 2	8
067	4 4	8
116	4 4	8
119	4 4	8
124	4 4	8
131	4 4	8
171	44	8
178	4 4	8
186	4 4	8
193	4 4	8
215	44	8
220	4 4	8
234	4 4	8
248	44	8
255	44	8
258	44	8
079	4 3	7
213	4 3	7
101	4 2	6
108	42	6
173	42	6
250	2 4	6
069	3 2	5
088	4 1	5
114	4 1	5
132	1 4	5
240	4 1	5
004	4	4
005	4	4
008	4	4
012	4	4
016	4	4
020	4	4
026	4	4
027	4	4
028	4	4
029	4	4
034	4	4
045	4	4
	9001111991 1880122027 7786499548 AB	

	9001111991 1880122027 7786499548 AB	
080	4	4
092	4	4
104	4	4
105	4	4
115	4	4
118	4	4
122	4	4
123	4	4
126	4	4
127	4	4
134	4	4
135	4	4
140	4	4
147	4	4
150	4	4
151	4	4
175	4	4
177	4	4
180	4	4
181	4	4
184	4	4
185	4	4
187	4	4
188	4	4
194	4	4
195	4	4
200	4	4
204	4	4
205	4	4
206	4	4
209	4	4
216	4	4
218	4	4
222	4	4
224	4	4
237	4	4
242	4	4
251	4	4
013	3	3
019	3	3
073	3	3
094	1 2	3
121	3	3
153	3	3
166	3	3
172	3	3
197	3	3
221	3	3
017	2	2
032	2	2
033	2	2
036	2	2
065	2	2
128	2	2
138	2	2
148	2	2
167	2	2
	9001111991 1880122027 7786499548 AB	

Table 4.4

SP 4

1.	917	-									
2.	087	283	-								
3.	088	345	160	-							
4.	106	444	182	698	-						
5.	114	353	144	762	705	-					
6.	129A	074*	065*	236	210	227	-				
7.	129B	044*	128*	450	404	393	496	-			
8.	905	054*	088*	410	346	373	340	448	-		
9.	924	046*	095*	532	468	469	N/A	717	577	-	
10.	178	043	089*	429	N/A	312	274	462	381	616	-
		9	0	0	1	1	1	1	9	9	1
		1	8	8	0	1	2	2	0	2	7
		7	7	8	6	4	9	9	5	4	8
							A	B			
		1.	2.	3.	4.	5.	6.	7.	8.	9.	10.

specificity. I have called this specificity 4A. Sera 129A, 129B, 905 and 924 detect the second specificity. I have called this SP4B. Three sera, 088, 106 and 114 detect both specificities. The final serum (178) detects some, but not all, of the second specificity. This may be a consequence of working near the end-point. Specificity 4A is defined by sera 087, 917, 088, 106 and 114 (Table 3.4A). For an animal to be scored as positive, it must react with four of the five sera. This criterion alone does not clearly distinguish between 4A and 4B as three sera (088, 106 and 114) will also react with the 4B specificity.

If my interpretation of this serograph is correct, sera 087 and 917 have very long tails. This result implies that the specificities detected by the tails could occur rarely and purely by chance amongst 4B+ve animals. I believe that full-siblings 062 and 063 reflect this chance occurrence. The mother, animal 061, is only positive for sera 917 and 087. She is therefore in the tails of 087 and 917 and is negative for 4A and 4B. The father, ram 025, is negative for sera 087 and 917 but positive with seven of the remaining eight sera. Therefore, he is 4B positive but 4A negative. The lambs 062 and 063 react with all eight sera. Presumably the 4B specificity is inherited from the father. This ensures that they react with eight of the ten sera. The maternal inheritance ensures that they react with sera 087 and 917. Therefore they react with all sera even in the absence of the 4A specificity. To prevent false assignment, specificity 4A is only assigned in the absence of specificity 4B.

TABLE 3.4A

SEROGRAPH OF SPECIFICITY SP4A

09011
81801
77864

038	44444	20
039	44444	20
040	44444	20
041	44444	20
042	44444	20
043	44444	20
044	44444	20
062	44444	20
063	44444	20
066	44444	20
129	44444	20
136	44444	20
189	44444	20
202	44444	20
225	44444	20
227	44444	20
228	44444	20
230	44444	20
231	44444	20
252	44444	20
201	44144	17
024	4444	16
099	4444	16
182	4 444	16
183	4 444	16
203	4444	16
245	4444	16
246	4444	16
253	4444	16
060	2 444	14
106	44 42	14
229	4442	14
006	44 4	12
025	444	12
037	4 44	12
075	444	12
090	444	12
100	444	12
110	444	12
111	444	12
112	444	12
120	444	12
142	444	12
143	444	12
144	444	12
145	444	12
146	444	12
154	444	12
156	444	12
161	444	12
168	444	12
239	44 4	12
243	44 4	12
254	44 4	12
257	44 3	11
247	24 4	10
093	1 44	9
107	414	9
023	44	8
031	4 4	8
052	44	8
061	44	8
067	4 4	8
071	44	8

09011
81801
77864

09011
81801
77864

078	4 4	8
116	4 4	8
152	44	8
160	4 4	8
249	44	8
255	44	8
256	4 4	8
258	44	8
064	24	6
068	24	6
004	4	4
005	4	4
008	4	4
012	4	4
016	4	4
020	4	4
026	4	4
027	4	4
028	4	4
029	4	4
034	4	4
079	4	4
080	4	4
088	4	4
104	4	4
105	4	4
109	4	4
114	4	4
115	4	4
118	4	4
119	4	4
122	4	4
123	4	4
124	4	4
131	4	4
147	4	4
178	4	4
185	4	4
186	4	4
187	4	4
193	4	4
194	4	4
195	4	4
200	4	4
206	4	4
213	4	4
216	4	4
217	4	4
218	4	4
220	4	4
237	4	4
242	4	4
244	4	4
013	3	3
019	3	3
069	3	3
073	3	3
121	3	3
166	3	3
197	3	3
238	3	3
017	2	2
032	2	2
036	2	2
128	2	2
138	2	2

09011
81801
77864

As alluded to earlier, eight sera detect the 4B specificity. Serum 178 has not been used in the antigen definition. I have called animals reacting with five of the seven sera positive (Table 3.4B). The inheritance of the 4A and 4B specificities is presented in Tables 5.4A and 5.4B.

These two tables show quite clearly that both specificities are inherited as autosomal dominants. Further, the family studies add further weight to the hypothesis that 4A and 4B are two distinct specificities.

The 4A specificity was present in four Scottish Blackface sera, and one Clun Forest serum. Sheep carrying this specificity came from several breeds. These included the East Friesland, Damline, Oxford Down, Tasmanian Merino and the Border Leicester. The 4B specificity was contained in eight sera. Three of the sera came from Scottish Blackface, one from a German Merino Landrace, one from a Finnish Landrace and two from a Finnish Landrace x Dorset Horn cross. In my panel, the specificity was only present in Damlines and Southdowns. Nonetheless, the presence of the antibody in several breeds (where the immunising stimulus almost certainly came from animals of the same breed) indicates that the specificity is likely to occur in many sheep breeds of diverse origin.

SP 5

There are only two sera in this cluster (Table 2.5). Their reactions are presented in Table 3.5. I have decided to treat serum 174 as the serum defining the specificity and serum 016 as a serum which detects only part of the specificity. Both sera were inherited in families.

Table 5.4A

Inheritance of Specificity SP 4A

Parents				Offspring					
Phenotype		Number		Phenotypes				Totals (M+F)	
R	E	R	E	M		I			
				+	-	+	-	+	-
+	+	0	0	-	-	-	-	-	-
+	-	0	0	-	-	-	-	-	-
-	+	6	10	1	5	4	2	5	7
-	-	10	48	0	35	0	50	0	85
TOTALS		11	58	1	40	4	52	5	92

TABLE 3.4B

SEROGRAPH OF SPECIFICITY SP4B

1199011		
2202801		
9954864		
AB		
024	4444444	28
025	4444444	28
062	4444444	28
063	4444444	28
075	4444444	28
099	4444444	28
100	4444444	28
110	4444444	28
111	4444444	28
112	4444444	28
120	4444444	28
142	4444444	28
143	4444444	28
145	4444444	28
146	4444444	28
154	4444444	28
161	4444444	28
107	4444414	25
071	444444	24
090	44 4444	24
093	444444	24
144	444 444	24
152	4444 44	24
156	444 444	24
168	44 4444	24
052	4 44 44	20
078	44444	20
006	4 4 4 4	16
042	4 444	16
043	4 444	16
182	4 444	16
183	4 444	16
227	4 444	16
252	4 444	16
038	2 444	14
228	2 444	14
229	4 442	14
039	444	12
040	444	12
041	444	12
044	444	12
059	444	12
066	444	12
129	444	12
136	444	12
189	444	12
202	444	12
203	444	12
225	444	12
230	444	12
231	444	12
247	44 4	12
253	444	12
256	3 4 4	11
037	2 44	10
201	144	9
233	4 41	9
031	4 4	8
091	4 4	8
109	4 4	8
171	44	8
215	44	8
217	44	8

1199011		
2202801		
9954864		
AB		
234	4 4	8
238	44	8
239	4 4	8
245	44	8
246	44	8
248	44	8
254	4 4	8
068	24	6
101	4 2	6
106	42	6
108	42	6
173	42	6
244	2 4	6
250	2 4	6
069	2 3	5
132	4 1	5
240	4 1	5
028	4	4
045	4	4
067	4	4
092	4	4
115	4	4
116	4	4
119	4	4
124	4	4
126	4	4
127	4	4
131	4	4
134	4	4
135	4	4
140	4	4
150	4	4
151	4	4
160	4	4
175	4	4
177	4	4
178	4	4
180	4	4
181	4	4
184	4	4
186	4	4
188	4	4
193	4	4
200	4	4
204	4	4
205	4	4
209	4	4
220	4	4
222	4	4
224	4	4
243	4	4
251	4	4
079	3	3
094	2 1	3
153	3	3
172	3	3
213	3	3
221	3	3
257	3	3
033	2	2
064	2	2
065	2	2
148	2	2
167	2	2
249	2	2

1199011
2202801
9954864
AB

Table 5.4B

Inheritance of Specificity SP 4B

Parents				Offspring					
Phenotype		Number		Phenotypes				Totals (M+F)	
R	E	R	E	M		F			
				+	-	+	-	+	-
+	+	1	1	1	1	0	1	1	2
+	-	2	15	5	7	7	14	12	21
-	+	3	4	1	1	5	2	6	3
-	-	9	38	0	25	0	27	0	52
TOTALS		11	58	7	34	12	44	19	78

Table 2.5

Sera Defining Specificity SP 5

Serum Identity	Breed of Origin	Country of Origin	Laboratory Designation	Immune (I) or Parous (P)	Test Dilution
174	D	Scotland	7J490	P	N
016*	SB	Scotland	1N135	P	N

TABLE 3.5

SEROGRAPH OF SPECIFICITY SP5

	10
	71
	46
064	44
067	44
106	44
160	44
245	44
246	44
254	44
255	44
256	44
257	44
258	44
029	4
031	4
045	4
052	4
068	4
113	4
114	4
116	4
120	4
121	4
122	4
123	4
124	4
177	4
188	4
244	4
250	4
252	4
032	2
---	---
041	3
	10
	71
	46

Table 4.5

SP 5

1.	174	-	
2.	016	566	-
		1	0
		7	1
		4	6
		1.	2.

However, it seems wisest to wait for further evidence before saying that serum 016 is a 'split' of serum 174. The SP 5 specificity is inherited as a mendelian dominant (Table 5.5).

The two sera 016 and 174 came from the Scottish Blackface and Damline breeds. The positive animals came from a variety of breeds including Damline, Border Leicester, Southdown, Oxford, East Friesland and Dorset Horn.

SP 6

There are twenty-two sera in this cluster (Table 2.6). The serograph is presented in Table 3.6. The reaction distribution of sheep against the twenty-two sera is given in figure 2. Twenty-two sheep have been excluded from figure 2 as they were not tested against all sera.

There are no animals which react with twelve sera. I have called sheep which react with thirteen or more sera positive. Most of the missed reactions are attributable to a few sera. Furthermore, these sera had several weak positive reactions, suggesting that the negative reactions were due to the serum being used near its end-point.

The inheritance of the SP 6 specificity is given in Table 5.6. In common with the other specificities, this is inherited as an autosomal dominant. Although common, it is present in only a few families. Many of the positives were Oxford Down x Texel crosses. The mothers and daughters were tested but unfortunately the ram was unavailable for family studies. The sera came from a variety of breeds including Southdown, Oxford Down, Border Leicester, Finnish Landrace x Dorset Horn, Damline, German Merino, Landrace and Clun Forest. The specificity was detected in Oxford Down, Damline, Finnish Landrace, Border Leicester and Southdown animals.

Table 5.5

Inheritance of Specificity SP 5

Parents				Offspring					
Phenotype R	E	Number R	E	Phenotypes M		F		Totals (M+F)	
				+	-	+	-	+	-
+ x +		0	0	-	-	-	-	-	-
+ x -		1	1	0	0	1	0	1	0
- x +		6	11	3	6	2	6	5	12
- x -		10	46	0	32	0	47	0	79
TOTALS		11	58	3	38	3	53	6	91

Figure 2

Reactions of 236 Sheep Against 22 Sera of the SP 6 Cluster

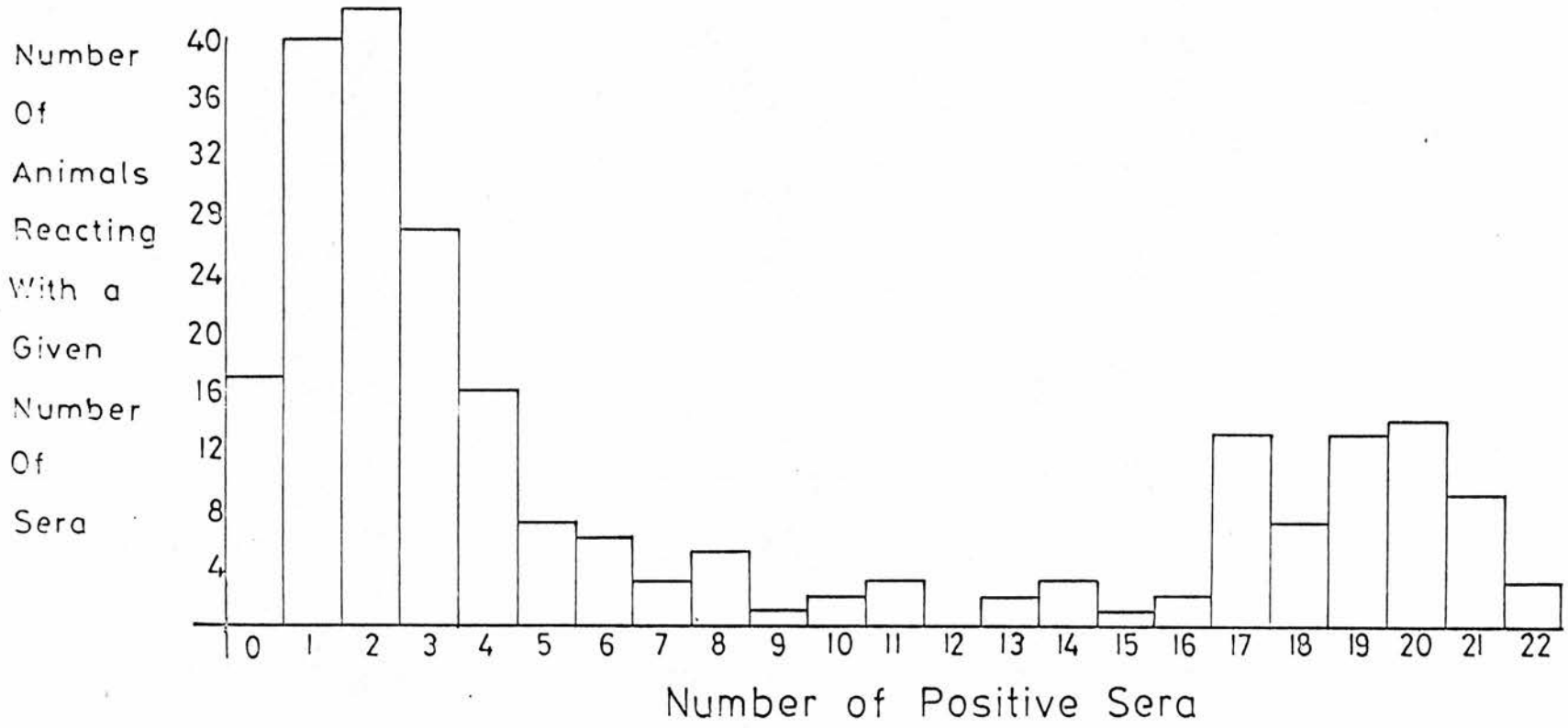


Table 2.6

Sera Defining Specificity SP 6

Serum Identity	Breed of Origin	Country of Origin	Laboratory Designation	Immune (I) or Parous (P)	Test Dilution
226	FD	Scotland	4FD15 25.5.78	I	1/16
211	O	Scotland	5Z4 15.12.77	P	1/4
213	O	Scotland	5Z8 18.12.77	P	N
231	SD	Scotland	5S22 5.1.78	P	1/2
910	GML	W. Germany	9114	I	1/2
157	FD	Scotland	7G325 13.2.78	I	1/2
156A	FD	Scotland	7G323 18.2.78	I	1/2
212	O	Scotland	5Z6 4.12.77	P	N
236	BL	Scotland	5L17 9.2.78	I	1/16
225	FD	Scotland	4FD15 9.2.78	I	N
127	FD	Scotland	64486 27.2.78	P	1/4
169	D	Scotland	7J447 3.4.78	P	1/8
200	O	Scotland	5Z8 10.8.77	P	N
128	FD	Scotland	64481 27.2.78	P	N
240	BL	Scotland	5L22 26.5.78	I	N
904	GML	W. Germany	9758	I	1/8
199	O	Scotland	5Z4 10.8.77	P	N
237	BL	Scotland	5L17 16.5.78	I	1/2
105	SB	Scotland	2T389	P	1/8
916	CF	England	140/1	I	1/2
032	SB	Scotland	1T188	P	N
156B	FD	Scotland	7G323 13.2.78	I	1/8

```
    2222911222112129121901
    2113155132260240930135
    6131076265790804975626
          A              B
004 4444444444444444444443  87
194 444444444444444434444   87
216 444444444444444444434   87
113 44444444444444444 444   84
114 44444444444444444 444   84
120 44444444444444444 444   84
195 44444444444444 4444444  84
206 444444444444444444 4    84
208 44444444444444444444    84
218 444444444444444444 4    84
244 44444444444444 444444   84
245 4444 4444444444444444   84
217 444444444444444444 144  81
002 444444444444444 444 44  80
037 44444444444444444 44    80
039 444444444444444444 44   80
040 444444444444444444 44   80
197 44444444444444444 4 4   80
198 444444444444444 444 44  80
210 444444444444444444 4 4  80
214 44444444444444444 4 4   80
036 444443444444444444 44   79
104 4444444444444444444434  79
123 44444444444444444  43   79
058 4444444444444444244     78
050 4444444444444444243     77
122 444444444144444 44 444  77
007 4444444444444444 23 34  76
026 444444444444444 44 44   76
111 44444444444444444  4    76
124 44444444444 444444  44  76
186 444444444444444 44 4 4  76
200 4444444444444444444     76
213 44444444444444444 4     76
008 444444444 44444 4443 4  75
121 4444444 4444444 44 434  75
003 444144441444444444 4    74
030 44444444444444442444    74
049 4444444444444444424     74
221 444444444444444444      74
017 4 444444444444441 44 44 73
105 4444444444444444344 2   73
011 444444444444444 42  2   72
048 44444444444444444 4     72
051 4444444444444444 4      72
112 444444444444444 4  44   72
185 44 4444 444 444 444444  72
188 44444444444444444   4   72
190 4444444444444444    4   72
202 444444444444442444 4 4  70
035 44444 4444 4444444 4    68
053 4444444444444  4444     68
096 444444444444444 4   4   68
184 444444 4444444444 4     68
189 44444444444444 4 4      68
193 444444444444 44  4 44   68
205 444 4444444444444 4     68
207 44444 444444444444      68
211 444444444444444 4 4     68
212 44444444444444444       68
219 4444444444444444        68
013 444444444444444  42      66
196 44 444444444444 4 4  2  66
056 414444344444414441 43   65
191 44444444444444144       65
164 44444 444444444444      64
179 4444444444444444        64
192 441444444444444 4       61
204 4444444 44444444        60
201 414144444444414 44 4    59
001 4 4 4444442444 2144     57
187 4444 4444 4444  4   4   56
199 44444444444444          56
031 444 44 44 44444  42      54
029 444 44 44 44444  4       52
059 44444  444  4 444  4     52
--------------------------------
116 444 4444  4 14    44     45
032 444 44 44  4444          44
110 444 444  4   4 4  4 4    44
150 444 44 4 4     4  444    44
151 444 4444 44       4      40
```

```
    2222911222112129121901
    2113155132260240930135
    6131076265790804975626
          A              B
028 4444  434     44 4       39
215 44442 4414        4  4   39
254 44  4 4      4 4   444   36
093 441 344  4   3 2  4       33
060 4   444   44 4 4          32
067 44 44 4 4    44           32
106 44  424      4 4    42    32
246 44   44      4     444    32
005     344   44 4    4  3    30
095 4   444    4   4    4     28
027 44   4   4   42     4     26
045 44  4 4   4    4          24
069 442 2 2 42    4           24
109 4   4      4 4 4 4         24
115 444 4  4          4        24
153 44   44  4        4        24
068 4   4 2      3 4    4      21
090 442  2   2   3     4       21
249 41   4      4     44       21
087 4   4     4    4    4      20
160 4   4        4     44      20
255 44    4      4      4      20
258 44    4      4      4      20
147 4   4          4   43      19
248 42    4              44    18
256 42    3      4       4     17
064 4   4        4     4       16
088 4   4          4     4     16
091     44       4      4      16
097 4   44         4            16
128 4   4          4     4      16
135     4          4    44      16
148 4   4          4     4      16
250 4   44               4      16
086 4   43         4              15
094 4   3          4     4      15
142 4   3          4     4      15
138 4   2          4     4      14
155     42             44       14
175 4   42         4              14
228 4   4        2    4          14
083     43         4    2        13
125 4   4          4  1          13
173       4 1      4     4       13
033       4           4 4        12
052 4   44                       12
066 4   4          4             12
072     42              24       12
073 4   4               4        12
074 4   4          4             12
075 4   4          4             12
078 4                    4 4      12
100     4          4     4       12
118 4   4          4             12
127 4   4          4             12
141 4   4          4             12
167 4   4          4             12
169 4   4          4             12
170 4   4          4             12
174 4   4          4             12
229 4   4 4                      12
238       44               4     12
241     4          4     4       12
242 4            4       4      12
247 4     4                4     12
251 4   4                  4     12
257 4            4       4      12
081 4   3          4              11
080 4   4     2                   10
117 4   2          4              10
237       4      2     4          10
085     14           4             9
234 4                    4 1      9
006  4  4                          8
015     44                         8
024     4                    4     8
025                  4     4      8
034 4   4                          8
043     4          4               8
047 4                    4         8
054     44                         8
```

```
    2222911222112129121901
    2113155132260240930135
    6131076265790804975626
          A              B
057 4                    4      8
079 4   4                       8
098     4          4            8
099     4                   4    8
101     4          4            8
119 4                       4    8
126     4                   4    8
129     44                       8
131 4   4                        8
132 4              4             8
133     4          4             8
157  4     4                     8
162 4              4             8
163 4              4             8
166 4              4             8
171       4      4               8
222 4     4                      8
224 4                    4      8
225 4   4                        8
226 4                    4      8
227 4   4                        8
230 4                    4      8
231 4   4                        8
232 4                    4      8
233     4                   4    8
235 4   4                        8
236 4   4                        8
239 4   2        2               8
252     4                   4    8
253 4   4                        8
014 3   4                        7
055     3                4       7
136     4                   3    7
154     4                   3    7
062 4         1              1   6
063 4         1              1   6
010 3   2                        5
061 4                       1    5
103       32                     5
240  1  4                        5
020 4                            4
022     4                        4
023 4                            4
038     4                        4
042     4                        4
044     4                        4
065     4                        4
071 4                            4
076     4                        4
082     4                        4
092                         4    4
102 4                            4
107       22                     4
108              4               4
130       4                      4
134              4               4
140              4               4
143                         4    4
145     4                        4
146                         4    4
149     4                        4
152              4               4
156                         4    4
158     4                        4
159     4                        4
161     4                        4
172              4               4
176     4                        4
177                         4    4
178 4                            4
180 4                            4
181 4                            4
182 4                            4
183 4                            4
203       4                      4
209       4                      4
220       4                      4
012  1  2                        3
041     3                        3
139       3                      3
046       2                      2
```

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    2222911222112129121901
```

```
    2222911222112129121901
```

Table 4.6

SP 6

1.	226	-																					
2.	211	385	-																				
3.	213	N/A	705	-																			
4.	231	347	661	720	-																		
5.	910	238	328	403	384	-																	
6.	157	212	474	507	454	259	-																
7.	156A	341	618	576	586	275	482	-															
8.	212	362	685	777	715	387	533	585	-														
9.	236	N/A	684	763	777	396	538	631	754	-													
10.	225	N/A	683	740	713	876	557	625	692	721	-												
11.	127	362	627	703	700	423	575	628	709	729	677	-											
12.	169	355	612	716	711	410	576	613	722	758	673	752	-										
13.	200	N/A	666	758	762	402	570	607	764	N/A	723	764	779	-									
14.	128	390	616	590	572	333	511	580	565	610	575	606	628	622	-								
15.	240	336	656	695	728	369	513	579	685	768	688	670	691	744	619	-							
16.	904	233	216	287	277	351	173	240	272	277	298	272	291	285	214	243	-						
17.	199	N/A	N/A	688	753	352	512	592	708	N/A	706	708	712	N/A	603	694	329	-					
18.	237	N/A	538	627	637	308	475	518	622	661	581	622	640	663	545	633	304	597	-				
19.	105	253	349	406	318	118	353	329	392	345	429	392	355	363	355	294	092	325	311	-			
20.	916	237	422	426	434	267	343	346	419	406	392	380	376	414	409	405	187	449	504	121	-		
21.	032	068	153	100	122	071	152	144	084	094	123	114	100	117	196	124	068	150	177	032	233	-	
22.	156B	189	383	329	414	187	332	N/A	339	392	353	383	352	392	375	361	121	379	484	232	374	242	-
		2	2	2	2	9	1	1	2	2	2	1	1	2	1	2	9	1	2	1	9	0	1
		2	1	1	3	1	5	5	1	3	2	2	6	0	2	4	0	9	3	0	1	3	5
		6	1	3	1	0	7	6 A	2	6	5	7	9	0	8	0	4	9	7	5	6	2	6
		1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.	13.	14.	15.	16.	17.	18.	19.	20.	21.	22.

Table 5.6

Inheritance of Specificity SP 6

Parents		Offspring		
Phenotype R E	Number R E	Phenotypes M	F	Totals (M+F)
		+ : -	+ : -	+ : -
+ x +	2 2	1 0	2 0	3 0
+ x -	1 9	2 5	5 4	7 9
- x +	3 8	2 2	3 5	5 7
- x -	9 39	0 29	0 37	0 66
TOTALS	11 58	5 35	10 46	15 82

SP 7

There are ten sera in this cluster (Table 2.7). The serograph is in Table 3.7. An animal must react with eight or more of the ten sera to be considered positive. The inheritance of the specificity is given in Table 5.7.

SP 7 was present in very few families and was rare in certain breeds but common in others. Unfortunately, in the breeds in which it was common the rams could not be typed. In the families studied, the observed segregation between mother and offspring was consistent with control by a mendelian dominant gene. Of course, in the absence of the ram, this could not be proved. Of the families that could be typed, two contained positive parents (Table 5.7). Despite the small numbers, the figures exclude control by a sex-linked recessive gene. As they are compatible with an autosomal dominant mode of inheritance, I shall assume this to be the case.

The sera came from a variety of breeds. These include Welsh Mountain, Damline, Finnish Landrace x Dorset Horn and German Merino Landrace. Positive sheep came from a variety of breeds including Dorset Horn, Damline, Border Leicester, Southdown and Oxford Down.

SP 8

This cluster contains three sera. (Table 2.8). Serum 017 is contained within serum 048. In turn, serum 048 is almost completely contained within serum 063. Only three animals react with serum 017. These are animals 029, 031 and 032. Animal 029 is the offspring of 031. While animal 032 is the full-sibling of animal 031. Serum 048 is also inherited in families. Serum 063 does not give a regular pattern/

Table 2.7

Sera Defining Specificity SP 7

Serum Identity	Breed of Origin	Country of Origin	Laboratory Designation	Immune (I) or Parous (P)	Test Dilution
151	FD	Scotland	64525 13.2.78	P	1/8
175	D	Scotland	7J506 18.5.78	P	1/4
173	D	Scotland	7J177 11.5.78	P	1/8
155	FD	Scotland	7G306 6.2.78	I	1/4
195	WM	Scotland	3W4 18.7.78	P	1/4
907	GML	W. Germany	9711	I	1/8
913	GML	W. Germany	9707	I	1/4
209	SB	Scotland	S197 6.6.77	P	1/2
115	SB	Scotland	2T275 25.7.78	P	1/2
153	FD	Scotland	64427 13.2.78	P	1/2

TABLE 3.7

SEROGRAPH OF SPECIFICITY SP7

1111199211 5775901015 1535573953		
029	4444444444	40
031	4444444444	40
032	4444444444	40
033	4444444444	40
046	4444444444	40
047	4444444444	40
050	4444444444	40
051	4444444444	40
052	4444444444	40
054	4444444444	40
055	4444444444	40
058	4444444444	40
180	4444444444	40
181	4444444444	40
200	4444444444	40
203	4444444444	40
211	4444444444	40
219	4444444444	40
220	4444444444	40
190	4444444443	39
049	444444444	36
179	4444444 44	36
187	4444 44444	36
192	44444444 4	36
209	4444444 44	36
237	4 44444444	36
238	44 4444444	36
044	44 444444	32
053	4 4444444	32
199	44 44444 4	32
038	44244444	30
223	44 244444	30
037	44 44444	28
039	44 44444	28
040	44 44444	28
041	4444 444	28
136	44 4 4444	28
043	4444 4 4	24
129	44 4444	24
042	4 43444	23
246	44 444 2	22
112	24 4244	20
175	4 444 4	20
247	4 4 444	20
248	4 4 444	20
252	44 4 44	20
253	44 4 4 4	20
059	4 4344	19
066	3 4 444	19
026	2 4 44 4	18
109	4 4442	18
173	1 4444	17
116	4 444	16
171	4 444	16
233	4 4 44	16
242	4444	16
250	4444	16
258	4444	16
027	4442	14
074	2 44 4	14
093	1 1 444	14
021	3 2 4 4	13
202	44 14	13
251	4441	13
024	4 4 4	12
060	4 44	12
084	4 4 4	12
1111199211		

1111199211 5775901015 1535573953		
089	1 434	12
096	1 4 34	12
097	44 4	12
110	4 44	12
113	4 44	12
114	444	12
127	444	12
168	444	12
169	444	12
170	44 4	12
226	4 44	12
228	4 44	12
232	4 44	12
088	4421	11
095	1 244	11
099	42 4	10
120	2 44	10
148	244	10
155	4 3 3	10
163	424	10
091	1 44	9
254	44 1	9
002	4 4	8
003	4 4	8
006	4 4	8
025	44	8
028	44	8
045	4 4	8
072	4 4	8
087	44	8
092	44	8
094	4 4	8
098	4 4	8
102	4 4	8
107	4 4	8
108	44	8
123	44	8
124	44	8
126	44	8
128	44	8
132	4 4	8
138	4 4	8
139	4 4	8
140	44	8
142	44	8
144	4 4	8
151	44	8
165	4 4	8
172	44	8
178	4 4	8
186	4 4	8
215	4 4	8
224	4 4	8
234	4 4	8
255	44	8
257	4 4	8
004	3 4	7
068	3 4	7
103	3 4	7
106	4 3	7
218	3 4	7
125	1 41	6
137	4 2	6
147	2 4	6
164	2 4	6
183	2 4	6
225	24	6
1111199211		

1111199211 5775901015 1535573953		
229	2 4	6
244	24	6
056	41	5
071	2 3	5
212	1 4	5
020	4	4
030	4	4
035	4	4
036	4	4
057	4	4
067	4	4
069	4	4
075	4	4
078	4	4
079	4	4
080	4	4
081	4	4
086	4	4
090	4	4
100	4	4
101	4	4
111	4	4
115	4	4
117	4	4
122	4	4
131	4	4
133	4	4
134	4	4
135	4	4
150	4	4
153	4	4
156	4	4
157	4	4
158	4	4
162	4	4
166	4	4
167	4	4
174	4	4
176	4	4
177	4	4
182	4	4
184	4	4
188	4	4
191	4	4
193	4	4
195	4	4
196	4	4
197	4	4
204	4	4
205	4	4
207	4	4
208	4	4
213	4	4
216	4	4
217	4	4
221	4	4
222	4	4
227	4	4
231	4	4
239	4	4
240	4	4
245	4	4
065	3	3
073	3	3
256	3	3
019	2	2
082	2	2
143	2	2
1111199211		

Table 4.7

SP 7

1.	151	-									
2.	175	472	-								
3.	173	402	645	-							
4.	155	455	458	356	-						
5.	195	467	571	545	379	-					
6.	907	522	487	443	373	454	-				
7.	913	485	490	482	331	536	672	-			
8.	209	341	268	233	111	355	463	509	-		
9.	115	165	287	307	188	297	253	258	167	-	
10.	153	482	387	447	306	357	326	379	288	161	-
		1	1	1	1	1	9	9	2	1	1
		5	7	7	5	9	0	1	0	1	5
		1	5	3	5	5	7	3	9	5	3
		1.	2.	3.	4.	5.	6.	7.	8.	9.	10.

Table 5.7

Inheritance of Specificity SP 7

Parents				Offspring					
Phenotype		Number		Phenotypes				Totals (M+F)	
R	E	R	E	M		F			
				+	-	+	-	+	-
+	+	0	0	-	-	-	-	-	-
+	-	1	1	0	0	1	0	1	0
-	+	1	1	1	0	0	0	1	0
-	-	10	56	0	40	0	55	0	95
TOTALS		11	58	1	40	1	55	2	95

Table 2.8

Sera Defining Specificity SP 8

Serum Identity	Breed of Origin	Country of Origin	Laboratory Designation	Immune (I) or Parous (P)	Test Dilution
048*	SB	Scotland	2N039	P	N
063	SB	Scotland	1N020	P	1/2
017*	SB	Scotland	1N154	P	N

TABLE 3.8

SEROGRAPH OF SPECIFICITY SP8

```
      000
      461
      837

029 444
031 444
032 444
171 44
173 44
233 44
247 44
248 44
-------
008 4
004  4
045  4
086  4
087  4
095  4
097  4
109  4
123  4
125  4
127  4
138  4
142  4
148  4
163  4
166  4
169  4
170  4
175  4
177  4
187  4
188  4
227  4
246  4
250  4
254  4
027  2
033  2
075  2
088  2
147  2
      000
      461
      837
```

Table 4.8

SP 8

048	-		
063	398	-	
017	N/A	N/A	-
	0	0	0
	4	6	1
	8	3	7
	1.	2.	3.

pattern of inheritance and has several weak positive reactions. This suggests that it may be detecting several specificities with some of the antibodies near the end-point. This would explain the irregular pattern of inheritance.

Sera 017 is also contained within clusters SP 5, SP 7, SP 8 and SP 10. In SP 7 it is unlikely that all seven sera each contain two antibodies. (One antibody being the same as the antibody in serum 017, the other reacting with the remainder of the sheep in the SP 7.) Therefore, I have not included serum 017 in the SP 7 cluster. Similar arguments apply to SP 5, SP 8, SP 9 and SP 10.

I have assumed that serum 048 is detecting a specificity which I call SP 8. I have not used serum 017 to define a specificity. As in SP 5, it seems wisest to wait for further evidence before 'splitting' SP 8. As animal 008 does not react with serum 063, I have not counted it as a positive.

The inheritance of specificity SP 8 is given in Table 5.8.

Sera 017, 048 and 063 all came from Scottish Blackface sheep. Serum 017 reacted with two Dorset rams and the daughter of one of them. Serum 048 reacted with an East Friesland ewe and her lamb, a Damline ewe and her lamb and also a Finnish Landrace ewe and a Tasmanian Merino ewe.

SP 9

There are six sera in this cluster (Table 2.9). I have taken animals which react with three or more sera as positive for the specificity (Table 3.9). With the exception of one positive animal/

Table 5.8

Inheritance of Specificity SP 8

Parents				Offspring					
Phenotype R		E		Number R	E	Phenotypes M		F		Totals (M+F)	
						+	-	+	-	+	-
+	x	+		0	0	-	-	-	-	-	-
+	x	-		1	1	0	0	1	0	1	0
-	x	+		3	3	1	1	1	1	2	2
-	x	-		10	54	0	39	0	53	0	92
TOTALS				11	58	1	40	2	54	3	94

Table 2.9

Sera Defining Specificity SP 9

Serum Identity	Breed of Origin	Country of Origin	Laboratory Designation	Immune (I) or Parous (P)	Test Dilution
911	GML	W. Germany	9115	I	1/2
145	FD	Scotland	64621	P	N
915	GML	W. Germany	9109	I	1/2
927	FD	Scotland	3E16 10.8.77	P	1/4
923	FL	Scotland	5L2092 24.11.79	I	1/8
204	O	Scotland	2Z31 8.8.77	P	N

TABLE 3.9

SEROGRAPH OF SPECIFICITY SP9

919992
141220
155734

Ø13	444444	24
Ø14	444444	24
Ø15	444444	24
Ø20	444444	24
Ø22	444444	24
Ø23	444444	24
Ø24	444444	24
Ø25	444444	24
Ø27	444444	24
Ø31	444444	24
Ø34	444444	24
Ø43	444444	24
Ø54	444444	24
Ø65	444444	24
Ø70	444444	24
Ø71	444444	24
Ø72	444444	24
Ø73	444444	24
Ø75	444444	24
Ø76	444444	24
Ø78	444444	24
Ø80	444444	24
Ø83	444444	24
Ø85	444444	24
Ø87	444444	24
100	444444	24
101	444444	24
102	444444	24
104	444444	24
105	444444	24
110	444444	24
116	444444	24
118	444444	24
125	444444	24
131	444444	24
133	444444	24
138	444444	24
158	444444	24
175	444444	24
177	444444	24
178	444444	24
180	444444	24
182	444444	24
184	444444	24
191	444444	24
193	444444	24
205	444444	24
207	444444	24
213	444444	24
227	444444	24
228	444444	24
235	444444	24
236	444444	24
250	444434	23
Ø05	343444	22
Ø49	444442	22
Ø55	444424	22
Ø98	444442	22
147	444442	22
Ø62	444144	21
Ø63	444144	21
Ø88	414444	21
Ø93	444441	21
136	443424	21
209	441444	21
Ø06	44444	20
Ø12	444 44	20
Ø16	44 444	20
Ø17	44 444	20
Ø35	44444	20
Ø36	44444	20
Ø40	44444	20
Ø41	44444	20
Ø44	44 444	20

919992
141220
155734

Ø46	44 444	20
Ø47	4 4444	20
Ø50	44 444	20
Ø51	4444 4	20
Ø52	4444 4	20
Ø53	4444 4	20
Ø58	44 444	20
Ø61	444 44	20
Ø64	44444	20
Ø74	4444 4	20
Ø79	444 44	20
Ø82	4 4444	20
Ø86	4444 4	20
Ø90	4444 4	20
Ø94	4444 4	20
Ø97	4444 4	20
Ø99	44444	20
106	44444	20
109	44444	20
115	44444	20
119	44444	20
120	4444 4	20
124	44444	20
132	44444	20
135	44444	20
141	44444	20
145	44444	20
146	44444	20
148	44444	20
149	44444	20
150	44444	20
152	44444	20
154	44444	20
155	44444	20
159	44444	20
160	44444	20
161	44444	20
167	44444	20
176	44444	20
181	44 444	20
183	44444	20
189	44444	20
201	44444	20
202	44444	20
219	44444	20
220	44444	20
233	4444 4	20
239	44444	20
240	4444 4	20
241	44444	20
242	44444	20
243	44444	20
246	44444	20
248	44444	20
249	44444	20
252	44444	20
253	44444	20
254	44444	20
255	44444	20
257	44444	20
258	44444	20
Ø28	4444 3	19
Ø77	444 34	19
231	44434	19
247	44443	19
Ø38	44244	18
151	24444	18
203	44244	18
142	44441	17
168	44144	17
Ø04	4444	16
Ø11	44 44	16
Ø18	44 44	16
Ø19	44 44	16

919992
141220
155734

Ø29	4444	16
Ø32	444 4	16
Ø37	44 44	16
Ø42	4444	16
Ø45	4444	16
Ø59	444 4	16
Ø60	4444	16
Ø66	4444	16
107	44 4 4	16
112	444 4	16
117	4444	16
123	4444	16
127	4444	16
134	4444	16
140	4444	16
144	44 4 4	16
153	444 4	16
156	4444	16
163	4444	16
166	4 444	16
169	4444	16
170	4444	16
173	444 4	16
186	444 4	16
204	44 44	16
215	444 4	16
225	444 4	16
229	4444	16
230	444 4	16
245	4444	16
251	4444	16
256	4444	16
Ø10	44 34	15
179	4443	15
Ø08	442 4	14
Ø39	4424	14
143	4424	14
172	4244	14
222	44 24	14
Ø81	444 1	13
Ø57	4 4 4	12
Ø67	444	12
Ø68	444	12
Ø95	4 44	12
108	444	12
111	444	12
126	44 4	12
128	4 44	12
174	444	12
187	4 4 4	12
188	4 44	12
212	44 4	12
217	444	12
221	44 4	12
223	4 4 4	12
224	444	12
129	442	10

Ø33	4	4	8
Ø92	4	4	8
232	4	4	8
234	44		8
244	44		8
226	3	3	6
Ø07		4	4
Ø48		4	4
Ø56	3	1	4
Ø89		4	4
Ø91	4		4
Ø96		4	4
162	4		4
208	4		4
238		4	4
Ø09	2		2
Ø26		2	2

919992
141220
155734

Table 4.9

SP 9

1.	911	-					
2.	145	695	-				
3.	915	630	634	-			
4.	927	591	594	634	-		
5.	923	473	435	442	468		
6.	204	186	222	073	146	264	-
		9	1	9	9	9	2
		1	4	1	2	2	0
		1	5	5	7	3	4
		1.	2.	3.	4.	5.	6.

animal from negative parents, the specificity appears to be inherited as an autosomal dominant (Table 5.9). The exception was animal 163. This animal also showed an aberrant inheritance for specificity SP 3A. The fact that two different antigens are involved lends support to the belief that false parentage is the explanation.

The SP 9 specificity is very common. Many of the positive animals will be homozygotes. All the offspring of a homozygote will be positive for SP 9. This explains why the ratio of positive to negative offspring did not approximate 1:1. Despite the high frequency of this specificity, only six sera of the 120 detect it. This may be a consequence of the fact that with high frequency genes, the frequency of incompatible immunisations will be low. Consequently antisera will be relatively rare.

The SP 9 specificity is present in all breeds studied. The six sera came from the German Merino, Landrace, Oxford Down and Finnish Landrace and a Finnish Landrace x Dorset Horn cross.

SP 10

This specificity is defined by four sera (Table 2.10). All animals reacting with two or more sera are regarded as positive. Eighty-seven sheep possess the specificity. The serograph is presented in Table 3.10.

The inheritance of the specificity is given in Table 5.10. In common with the other specificities discussed earlier, the specificity appears to be inherited as an autosomal dominant.

Table 5.9

Inheritance of Specificity SP 9

Parents				Offspring					
Phenotype		Number		Phenotypes				Totals (M+F)	
R	E	R	E	M		F			
				+	-	+	-	+	-
+ x +		8	34	21	4	28	3	49	7
+ x -		5	8	4	0	8	1	12	1
- x +		2	15	7	5	11	3	18	8
- x -		1	1	0	0	1	1	1	1
TOTALS		11	58	32	9	48	8	80	17

Table 2.10

Sera Defining Specificity SP 10

Serum Identity	Breed of Origin	Country of Origin	Laboratory Designation	Immune (I) or Parous (P)	Test Dilution
908	GML	W. Germany	9509	I	1/2
918	SCBL	England	81B/7	I	1/2
208	SB	Scotland	2S17 18.7.77	P	1/2
921	CF	England	120/0	P	N

TABLE 3.10

SEROGRAPH OF SPECIFICITY SP10

9929
0102
8881

024	4444	16
033	4444	16
043	4444	16
045	4444	16
072	4444	16
109	4444	16
141	4444	16
147	4444	16
154	4444	16
155	4444	16
158	4444	16
167	4444	16
176	4444	16
064	4443	15
073	4443	15
079	4443	15
005	3344	14
082	4442	14
076	4423	13
031	444	12
032	444	12
049	44 4	12
050	44 4	12
051	44 4	12
053	44 4	12
054	44 4	12
058	44 4	12
065	444	12
083	444	12
098	44 4	12
099	444	12
100	444	12
101	444	12
102	444	12
104	444	12
105	444	12
115	444	12
116	444	12
118	444	12
119	444	12
131	444	12
132	444	12
133	444	12
135	444	12
145	444	12
149	444	12
150	444	12
152	444	12
159	444	12
160	444	12

9929
0102
8881

166	444	12
179	44 4	12
180	44 4	12
181	44 4	12
219	44 4	12
220	44 4	12
223	44 4	12
022	44 3	11
078	434	11
085	344	11
240	434	11
046	24 4	10
161	244	10
209	44 2	10
029	44	8
036	44	8
042	44	8
047	4 4	8
052	4 4	8
055	44	8
071	4 4	8
077	44	8
080	4 4	8
129	44	8
139	44	8
177	4 4	8
188	4 4	8
236	4 4	8
241	44	8
007	34	7
039	43	7
070	43	7
168	43	7
246	34	7
035	42	6
138	4 2	6
239	2 4	6
	---	---
006	4	4
015	4	4
020	4	4
021	4	4
027	4	4
028	4	4
037	4	4
038	4	4
040	4	4
041	4	4
044	4	4

9929
0102
8881

056	4	4
087	4	4
088	4	4
090	4	4
092	4	4
093	4	4
094	4	4
103	4	4
106	4	4
127	4	4
136	4	4
142	4	4
148	4	4
157	4	4
175	4	4
186	4	4
187	4	4
190	4	4
192	4	4
200	4	4
202	4	4
203	4	4
205	4	4
206	4	4
208	4	4
211	4	4
216	4	4
225	4	4
226	4	4
237	4	4
238	4	4
245	4	4
252	4	4
254	4	4
255	4	4
002	3	3
013	3	3
086	3	3
014	2	2
057	2	2
066	2	2
068	2	2
075	2	2
097	2	2
130	2	2
156	2	2
165	2	2
224	2	2
256	2	2

9929
0102
8881

Table 4.10

SP 10

1.	908	-			
2.	918	412	-		
3.	208	393	426	-	
4.	921	301	450	202	-
		9	9	2	9
		0	1	0	2
		8	8	8	1
		1.	2.	3.	4.

Table 5.10

Inheritance of Specificity SP 10

Parents				Offspring					
Phenotype R		E	Number R	E	Phenotypes M		F		Totals (M+F)
					+ : -		+ : -		+ : -
+	x	+	2	7	4	1	6	1	10 2
+	x	-	3	8	2	2	7	8	9 10
-	x	+	5	14	4	5	8	7	12 12
-	x	-	8	27	0	23	0	21	0 44
TOTALS			11	58	10	31	21	37	31 68

The sera defining the specificity came from a Scottish Blackface, a German Merino Landrace and a Suffolk x Cheviot x Border Leicester cross. The specificity was present in Finnish Landrace, Damlines, Oxford, Southdowns and Border Leicesters.

SP 11

This specificity is determined by a single serum (Table 2.11). The serum reacted with only eleven animals amongst the 258 tested. Three of the positive animals shared the same father. The three mothers were all negative. Unfortunately, the father could not be tested. Two of the remaining eight sheep were mother and daughter. The father was negative. The remaining six sheep were a father and all five of his offspring. The family results are summarised in Table 5.11.

The results presented here led me to the conclusion that the serum detected a rare specificity. The family material is small but it does suggest that the specificity is inherited as a mendelian dominant.

SP 12

There are three sera defining this specificity (Table 2.12). I have taken serum 047 as the defining serum, although serum 022 has a lower reaction frequency (Table 3.12). Serum 022 fails to react with four animals which serum 047 reacts with. In particular, serum 022 reacts with animal 218 but not with animals 215 or 216. Animal 215 is the mother/

Table 5.11

Inheritance of Specificity SP 11

Parents				Offspring					
Phenotype R	E	Number R	E	Phenotypes M		F		Totals (M+F)	
				+	-	+	-	+	-
+ x +		0	0	-	-	-	-	-	-
+ x -		1	5	4	0	1	0	5	0
- x +		1	1	0	1	1	0	1	1
- x -		10	52	0	36	0	54	0	90
TOTALS		11	58	4	37	2	54	6	91

Table 2.11

Sera Defining Specificity SP 11

Serum Identity	Breed of Origin	Country of Origin	Laboratory Designation	Immune (I) or Parous (P)	Test Dilution
185	FD	Scotland	23095 13.7.78	P	N

Table 2.12

Sera Defining Specificity SP 12

Serum Identity	Breed of Origin	Country of Origin	Laboratory Designation	Immune (I) or Parous (P)	Test Dilution
022*	SB	Scotland	1N181	P	N
047	SB	Scotland	2N019	P	N
187*	FD	Scotland	4G105 13.7.78	P	N

Table 2.13

Sera Defining Specificity SP 13

Serum Identity	Breed of Origin	Country of Origin	Laboratory Designation	Immune (I) or Parous (P)	Test Dilution
138A	FD	Scotland	64447 7.3.78	P	N
138B	FD	Scotland	64447 7.3.78	P	1/8

Table 2.14

Sera Defining Specificity SP 14

Serum Identity	Breed of Origin	Country of Origin	Laboratory Designation	Immune (I) or Parous (P)	Test Dilution
191	C	Scotland	4C27 20.7.78	P	1/8

TABLE 3.12

SEROGRAPH OF SPECIFICITY SP12

	010
	482
	772
028	444
067	444
069	444
113	444
115	444
218	444
196	44
197	44
215	44
216	44
---	---
146	2
154	2
024	4
108	4
142	4
143	4
161	4
195	4
206	2
	010
	482
	772

Table 4.12

SP 12

1.	022	-		
2.	047	656	-	
3.	187	490	N/A	-
		0	0	1
		2	4	8
		2	7	7
		1.	2.	3.

Table 5.12

Inheritance of Specificity SP12

Parents				Offspring					
Phenotype		Number		Phenotypes				Totals (M+F)	
R	E	R	E	M		F			
				+	-	+	-	+	-
+	+	0	0	-	-	-	-	-	-
+	-	0	0	-	-	-	-	-	-
-	+	2	2	1	1	1	1	2	2
-	-	11	56	0	39	0	54	0	93
TOTALS		11	58	1	40	1	55	2	95

mother of 216 and 218. The ram was not tested. It is possible that the ram has a specificity detected by 022 and not by 047 and 187. The ram had a total of 22 offspring. Serum 022 alone only reacts with three out of twenty-two lambs. This suggests that serum 022 is missing reactions.

Further, due to the extremely low reaction frequency of serum 022, I have not been able to titrate this serum. If the serum is being tested near the end-point this would explain the 'missed' reactions. The specificity has been found in only two families. The two families are both Damlines. The observed inheritance is presented in Table 5.12. The two families suggest an autosomal dominant mode of inheritance. As this is in agreement with the inheritance of the other specificities, I shall assume this to be the case.

Two of the sera were from Scottish Blackface animals and the third from a Finnish Landrace x Dorset Horn crossbreed. Positive animals were found in Damline and Oxford Down sheep.

SP 13

This specificity is defined by two dilutions of the same serum, 138A and 138B. The only complete family that could be typed was the Oxford family from Dryden. The ram, the three ewes and all three offspring were positive. An Oxford-Down x Texel family was also typed. It consisted of one Texel ram, eleven Oxford-Down ewes and twenty-two offspring. Unfortunately, the sire was not available for family studies. Five ewes were positive. Five of their eleven offspring were also positive. The other eleven lambs, which were

all/

Table 5.13

Inheritance of Specificity SP13

Parents				Offspring					
Phenotype R		E	Number R	E	Phenotypes M		F	Totals (M+F)	
					+ : -		+ : -	+ : -	
+	x	+	1	3	2	0	1 0	3	0
+	x	-	0	0	-	-	- -	-	-
-	x	+	0	0	-	-	- -	-	-
-	x	-	10	55	0	39	0 55	0	94
TOTALS			11	58	2	39	1 55	3	94

all negative, came from six negative dams. If the sire had been negative, this would suggest an autosomal dominant inheritance. Although, recessive inheritance cannot be excluded. If the inheritance is recessive, the sire must have been positive

The other dilution of this serum reacted with 253 out of 258 sheep tested. The serum came from a parous Finnish Landrace x Dorset Horn ewe. The only positive sheep detected by the more diluted reagent were Oxford Down ewes and their offspring.

SP 14

The SP 14 specificity is defined by a single serum (Table 2.14). The serum reacts with only four sheep. Three of the four sheep are an East Friesland ram and his two offspring. The two offspring came from two different ewes. Unfortunately, only one of the two mothers could be tested. She was negative. Although this single family is far from conclusive, I will regard the specificity as an autosomal dominant unless subsequent work makes this untenable.

The serum came from a Cheviot sheep. The four positive sheep came from the East Friesland and Cheviot breeds.

SP 15

This specificity is also defined by a single serum (Table 2.15). Of the 258 animals tested, the specificity reacts with only five sheep. Two of the five sheep are Damline ewes, one with a positive lamb and one with two out of three lambs positive. The two ewes had been mated/

Table 5.14

Inheritance of Specificity SF 14

Parents				Offspring					
Phenotype		Number		Phenotypes				Totals (M+F)	
R	E	R	E	M		F			
				+	-	+	-	+	-
+	x +	0	0	-	-	-	-	-	-
+	x -	1	1	0	0	1	0	1	0
-	x +	0	0	-	-	-	-	-	-
-	x -	10	57	0	41	0	55	0	96
TOTALS		11	58	0	41	1	55	1	96

Table 2.15

Serum Defining Specificity SP 15

Serum Identity	Breed of Origin	Country of Origin	Laboratory Designation	Immune (I) or Parous (P)	Test Dilution
906	GML	W. Germany	9751	I	N

mated to two different negative rams (Table 5.15). In this small study, the specificity appears to behave as an autosomal dominant.

The serum came from a German Merino Landrace. The positive animals were all Damline.

Unassigned Sera

One hundred and twelve sera were used for antigen definition. All the one hundred and twelve antisera were assigned to one of fifteen clusters. Six non-reactive sera were also put into the test to study the occurrence of false positive reactions. Five of the six sera were totally negative in this test. The sixth serum reacted with one animal (a ewe). The six non-reactive sera considered together suggest that false positives are very infrequent in this particular series of tests.

3. Independence of Specificities

In order to check that the specificities were not related to each other, I looked to see if there were any positive associations between them. Specificities were compared in pairs. For each pair, a 2 x 2 table was constructed. Chi-squares values (X^2), binary correlation coefficients (r) and probability of association (p) were calculated from the 2 x 2 table. Sub-group relationships (i.e. one serum completely contained within the other) and positive associations ($r > 0.205$; $p < 0.001$) were sought.

Specificities, 3A, 3B, 4A, 4B, 10, 13, 14 and 15 were all very nearly/

Table 5.15

Inheritance of Specificity SP 15

Parents		Offspring		
Phenotype R E	Number R E	Phenotypes M	F	Totals (M+F)
		+ : -	+ : -	+ : -
+ x +	0 0	- -	- -	- -
+ x -	0 0	- -	- -	- -
- x +	2 2	1 0	2 1	3 1
- x -	11 56	0 40	0 53	0 93
TOTALS	11 58	1 40	2 54	3 94

nearly, or completely, contained within specificity 9. This is illustrated in figure 3. Some specificities are very rare while SP 9 is very common. Therefore, some of the sub-group relationships may just have been chance occurrances.

As SP 9 contained the reactions of several other specificities and as SP 9 occurred at a very high frequency, it may be that SP 9 is actually composed of several specificities. Another possibility is that specificity 9 is actually a second antigenic determinant on the same molecule.

There were no other sub-group relationships. Only one pair of specificities showed a significant positive association. They were specificities SP 7 and SP 9 ($X^2 = 11.027$, $r = 0.207$, $p < 0.001$). The association has one of two explanations. Firstly, the specificities may define the same antigen. This is unlikely as both specificities are reasonably well-defined. Secondly, the two specificities may not be independently distributed in the population tested. The non-random distribution could reflect an underlying genetic mechanism (e.g. inbreeding or linkage disequilibrium). Alternatively, the association between SP 7 and SP 9 may just be a chance occurrence. A total of one hundred and thirty six comparisons were made. It is not too surprising that one comparison is significant.

The distribution of the specificities in the population is given in figure 4.

FIGURE 3

THE RELATIONSHIP OF SPECIFICITY SP9 TO THE OTHER SPECIFICITIES

	SSSSSSSSSS PPPPPPPPPP 0100001110 9034433547 AABB
098	444 4 4
045	444 4
109	444 4
101	444 2
005	444
116	444
118	444
132	444
133	444
135	444
138	444
141	444
147	444
166	444
241	444
043	44 4 4
239	44 4 4
006	44 4
039	44 4
042	44 4
129	44 4
246	44 4
100	44 44
152	44 44
099	44 4 4
052	44 4 4
024	44 4
071	44 4
078	44 4
145	44 4
154	44 4
161	44 4
168	44 4
036	44 4
083	44 4
085	44 4
150	44 4
180	44 4 4
181	44 4 4

```
     SSSSSSSSSS
     PPPPPPPPPP
     0100001110
     9034433547
       AABB
209 44     4  4
177 44     4
031 44      4 4
064 44      4
065 44      4
029 44        4
032 44        4
046 44        4
047 44        4
049 44        4
050 44        4
051 44        4
053 44        4
054 44        4
055 44        4
058 44        4
179 44        4
219 44        4
220 44        4
022 44
070 44
072 44
073 44
076 44
077 44
079 44
080 44
082 44
102 44
104 44
105 44
115 44
119 44
131 44
149 44
155 44
158 44
159 44
160 44
176 44
188 44
236 44
240 44
060 4 44
066 4 44
025 4 4 4
075 4 4 4
142 4 4 4
127 4 4    2
```

```
          SSSSSSSSS
          PPPPPPPPP
          0100001110
          9034433547
            AABB
028       4  4
074       4  4
081       4  4
086       4  4
087       4  4
088       4  4
094       4  4
095       4  4
097       4  4
108       4  4
117       4  4
123       4  4
124       4  4
125       4  4
128       4  4
134       4  4
140       4  4
148       4  4
151       4  4
153       4  4
163       4  4
169       4  4
170       4  4
172       4  4
173       4  4
174       4  4
175       4  4
182       4   4   4
183       4   4   4
038       4   4       4
044       4   4       4
203       4   4       4
040       4   4
041       4   4
136       4   4
189       4   4
201       4   4
202       4   4
225       4   4
227       4   4
228       4   4
229       4   4
230       4   4
231       4   4
245       4   4
247       4   4
252       4   4
253       4   4
254       4   4
```

```
          SSSSSSSSS
          PPPPPPPPP
          0100001110
          9034433547
            AABB
255 4   4
062 4     4
063 4     4
090 4     4
093 4     4
107 4     4
110 4     4
111 4     4
112 4     4
120 4     4
143 4     4
144 4     4
146 4     4
156 4     4
035 4      4
178 4       4
186 4       4
191 4       4
193 4       4
212 4       4
213 4       4
215 4       4
217 4       4
222 4       4
184 4       2
187 4         4 4
023 4           4
249 4           4
008 4
010 4
011 4
012 4
013 4
014 4
015 4
016 4
017 4
018 4
019 4
020 4
027 4
034 4
037 4
057 4
059 4
061 4
067 4
068 4
106 4
```

```
          SSSSSSSSSS
          PPPPPPPPPP
          0100001110
          9034433547
            AABB
126       4
204       4
205       4
207       4
221       4
224       4
233       4
235       4
242       4
243       4
244       4
248       4
250       4
251       4
256       4
257       4
258       4
167       4    4
033       4         4
223       4         4
007       4
139       4
004        4
190                 4
192                 4
199                 4
200                 4
211                 4
237                 4
238                 4
          SSSSSSSSSS
          PPPPPPPPPP
          0100001110
          9034433547
            AABB
```

FIGURE 4

THE DISTRIBUTION OF THE SPECIFICITIES IN THE POPULATION TESTED.

```
     SSSSSSSSSSSSSSSS
     PPPPPPPPPPPPPPPP
     0000000000011111
     1233445678901234 5
       ABAB
033  44       4  4
237  44       4
116  4 4   4    44
090  4     4    4
093  4     4    4
110  4     4    4
241   444        44
138   44         44
074   44         4
081   44         4
087   44         4
088   44         4
094   44         4
095   44         4
097   44         4
108   44         4
117   44         4
140   44         4
148   44         4
163   44         4
170   44         4
172   44         4
174   44         4
175   44         4
086   44         4
083   4 4        44
106   4  4 4     4
044   4  4    4  4
203   4  4    4  4
129   4  4       44
136   4  4       4
107   4   4      4
144   4   4      4
146   4   4      4
156   4   4      4
029   4     444444
114   4     44
068   4     4    4
257   4     4    4
```

```
     SSSSSSSSSSSSSSSS
     PPPPPPPPPPPPPPPP
     0000000000011111
     1233445678901234 5
       ABAB
187  4      44  4
190  4      44
192  4      44
199  4      44
200  4      44
211  4      44
007  4      4    4
001  4      4
002  4      4
026  4      4
030  4      4
096  4      4
003  4      4
209  4       4 44   4
220  4       4 44
171  4        4
065  4          44      4
022  4          44
070  4          44
072  4          44
076  4          44
077  4          44
082  4          44
102  4          44
119  4          44
149  4          44
155  4          44
158  4          44
159  4          44
176  4          44
240  4          44
126  4          4 4
010  4          4
012  4          4
020  4          4
139  4            4
069  4              4
009  4
021  4
084  4
089  4
091  4
092  4
103  4
130  4
137  4
157  4
165  4
045   44   4    44
```

```
      SSSSSSSSSSSSSSSS
      PPPPPPPPPPPPPPPP
      0000000000011111
      1233445678901234 5
        ABAB
098   44        44     4
109   44        44
060   4 4       4
066   4 4       4
025   4  4      4
075   4  4      4
142   4  4      4
123   4   44    4
124   4   44    4
004   4    4    4
173   4       44
101   4        44     4
005   4        44
118   4        44
132   4        44
133   4        44
135   4        44
141   4        44
147   4        44
166   4        44
167   4        44
127   4        4 4    4
028   4        4  4
125   4        4
128   4        4
134   4        4
151   4        4
153   4        4
169   4        4
043    44      44
100    4 4     44
152    4 4     44
036    4   4   44
035    4   4   44
085    4       44
150    4       44
245     4 44   4
246     4 4    44
252     4 4    4
039     4  4   44
040     4  4   4
189     4  4   4
202     4  4   4
201     4  4   4
038     4   4  4
042     4      44
228     4      4 4
230     4      4 4
182     4      4   4
```

```
     SSSSSSSSSSSSSSSS
     PPPPPPPPPPPPPPPP
     0000000000011111
     1233445678901234 5
       ABAB
183    4      4    4
253     4      4     4
041     4      4
225     4      4
227     4      4
229     4      4
231     4      4
120      444   4
052      44 4 44
111      4 4   4
112      4 4   4
099      4     44     4
024      4     44
071      4     44
078      4     44
145      4     44
154      4     44
161      4     44
168      4     44
062      4     4
063      4     4
143      4     4
031       444444     4
188       44   44
113       44       4
121       44
122       44
244       44
032       4 4444
177       4    44   4
064       4    44     4
160       4    44
067       4    4   4
254       4    4
255       4    4
250       4    4
256       4    4
258       4    4
049        44 44
050        44 44
051        44 44
053        44 44
058        44 44
179        44 44
219        44 44
104        4  44
105        4  44
186        4  4    4
191        4  4    4
```

```
SSSSSSSSSSSSSSSS
PPPPPPPPPPPPPPPP
0000000000011111
1233445678901234 5
  ABAB
193       4   4    4
212       4   4    4
213       4   4    4
217       4   4    4
184       4   4    4
008       4   4
011       4   4
013       4   4
017       4   4
037       4   4
059       4   4
204       4   4
205       4   4
207       4   4
221       4   4
196       4       4
197       4       4
216       4       4
218       4       4
048       4
056       4
164       4
185       4
194       4
195       4
198       4
206       4
208       4
210       4
214       4
047        4 444
055        4 444
180        4 44   4
181        4 44   4
046        4 44
054        4 44
223        4 44
238        4
247         44
233         44
248         44
239           444   4
115           44 4
073           44
079           44
080           44
131           44
236           44
057           4 4
```

```
     SSSSSSSSSSSSSSSS
     PPPPPPPPPPPPPPPP
     0000000000011111
     1233445678901234 5
       ABAB
224              4  4
215              4   44
178              4    4
222              4    4
023              4      4
249              4      4
006              4
061              4
014              4
015              4
016              4
018              4
019              4
027              4
034              4
235              4
242              4
243              4
251              4
226                 4
232                 4
234                 4
162
     SSSSSSSSSSSSSSSS
     PPPPPPPPPPPPPPPP
     0000000000011111
     1233445678901234 5
       ABAB
```

4. Gene Frequencies

The phenotypic and gene frequencies for the seventeen specificities were calculated. Data from different breeds was not pooled as breeds form separate breeding populations. Only one breed provided sufficient animals for a reliable estimate. This was the Damline breed. Only parents were used in the analysis. Ultimately, forty-six sheep were used to calculate gene frequencies. As far as is known, the forty-six sheep were not related, although a small proportion may have been half-siblings.

Gene frequencies (p) were calculated from the formula:-

$$p = 1 - \sqrt{(1-f)};$$

where f is the phenotypic frequency of the antigen.

Standard errors were calculated from -

$$S.E. = \sqrt{\left(\frac{p(1-p)}{2N}\right)}$$

where N is the total number of animals tested, and p remains the gene frequency.

Both formulae assume that the population is in Hardy-Weinberg equilibrium.

The results are summarised in Table 6. They show that some antigens are very rare while others are quite common.

Table 6

Gene Frequencies of the Specificities in Damline Sheep

Specificity	Total Sheep Tested (N)	Positive Sheep	Phenotypic Frequency (f)	Gene Frequency (P)	Standard Error
SP 1	46	3	0.065	0.033	0.019
SP 2	46	18	0.391	0.220	0.043
SP 3A	46	17	0.370	0.206	0.042
SP 3B	46	3	0.065	0.033	0.019
SP 4A	46	3	0.065	0.033	0.019
SP 4B	46	7	0.152	0.079	0.028
SP 5	46	8	0.174	0.091	0.030
SP 6	46	4	0.087	0.044	0.021
SP 7	46	0	-	-	-
SP 8	46	1	0.022	0.010	0.010
SP 9	46	39	0.848	0.601	0.038
SP 10	46	20	0.435	0.248	0.052
SP 11	46	0	-	-	-
SP 12	46	3	0.065	0.033	0.019
SP 13	46	0	-	-	-
SP 14	46	0	-	-	-
SP 15	46	2	0.043	0.022	0.015

Discussion

There are several unusual features of this analysis. They are discussed separately.

i) Each serum has only been assigned to one specificity, even though in several instances there was clear evidence that a serum reacted with two or more specificities. This was a deliberate decision. This was partly to simplify the analysis and partly to ensure that the sera in a cluster were at the correct dilution. Many of my sera contained several antibodies. It is possible that in some sera the antibodies present would have different titres. Running antisera at the optimal dilution for the highest titred antibody could mean that the other antibodies would be tested near the end-point. Thus, the extra antibodies could be less reliable than the main specificity. Therefore, no antiserum was assigned to more than one specificity.

ii) Any serum which showed significant positive associations with other sera was clustered. Often, a minimum size of correlation coefficient is demanded before a serum is admitted to a cluster. Bodmer et al. (1969) recommended a correlation coefficient of 0.4, while Mickey's analysis demands a minimum correlation coefficient of 0.3 (Bernoco, 1978 - personal communication). In this analysis, the only requirement was that the association was statistically significant. A probability of less than 0.01 is satisfied by any correlation coefficient greater than 0.160, while a probability of less/'

less than 0.001 is given by a correlation coefficient greater than 0.205. Sera which only react with part of a cluster need not necessarily give high correlation coefficients with other sera in the cluster. Nonetheless, these sera can be very informative. They may, for example, indicate the presence of several antigens in a specificity. Therefore, sera with low correlation coefficients were clustered. If minimum standard had been set, the results would have been much the same. Sera 920 (SP 2), 904 (SP 6) and serum 204 (SP 9) are the only sera which might have been excluded from their clusters.

When sera react with only part of a cluster, this could be due to a) working near the end-point or b) two or more different antigens being involved. In my test, if the sera involved gave irreproducible results, a proportion of weak positives and also if the sera were not inherited in families, this was taken to indicate working near the end-point. Generally, clusters were not split into two or more different groups unless there was compelling evidence to do so (e.g. two or more sera giving concordant reactions which are inherited in families).

Several sera appeared to be detecting only part of a specificity. This was initially surprising as the most likely explanation was working near the end-point. However, all sera were titrated before use and used two doubling dilutions before the end-point. There are three possible reasons to explain why sera may have been tested at the wrong dilution. It could be due to variations in test sensitivity/

sensitivity from day to day. However, results presented in Chapter 3 showed that test sensitivity did not show much variation between tests. Alternatively, testing sera two doubling dilutions before the end-point may be an inappropriate dilution for my test. However, titrations with sera, which were apparently directed against a single antigen, showed that the same sheep reacted at two and also at three dilutions above the end-point. There were, however, a small number of 'missed' reactions at the end-point itself and occasionally at one doubling dilution above the end-point. Therefore, two doubling dilutions above the end-point appears satisfactory. The final possibility is that the sera may have been titrated against animals of another specificity. If the specificity is rare or not present in the two hundred and fifty-three animals tested, it would not show up in the test. Of the one hundred and twelve sera used, nine (8%) were weak. They were 920 (SP 2), 156B (SP 6), 032 (SP 6), 916 (SP 6), 105 (SP 6), 923 (SP 9), 204 (SP 9), 921 (SP 10) and 022 (SP 12). Different explanations may be necessary for different sera.

iv) All sera were assigned to specificities. In part, this is due to the fact that all sera were titrated before use. In part, it is due to the fact that even sera with low correlation coefficients were assigned to clusters.

The sera came from a variety of breeds and countries including the German Merino Landrace, the prealpe from France, Finnish Landrace and both hill and down breeds from Scotland and England. Additionally, the majority of sera came from two flocks not represented in the sample/

sample of two hundred and fifty-three sheep. The two flocks were Scottish Blackface and the Finnish Landrace x Dorset Horn cross-breed. Nonetheless, the sera from these two flocks all reacted. Further, the animals tested came from a variety of sources, and all sheep, with only one exception (animal 162), were positive for at least one specificity. The twin observations that all sera reacted and nearly all sheep were positive suggests that the majority of lymphocyte antigens present in sheep were detected. This point will be considered further in the next chapter.

In summary, this chapter has shown that there are at least seventeen different lymphocyte antigens in sheep which appear to be inherited as mendelian dominants, with presence dominant to absence. They are present in a wide variety of breeds from diverse origins. The antigens are not necessarily detecting different alleles. Thus, several specificities may be sub-groups of SP 9. The relationships between the different antigens are discussed in the next chapter.

CHAPTER 6

THE GENETIC RELATIONSHIPS BETWEEN LYMPHOCYTE ANTIGENS

Introduction

Materials and Methods

1. Selection of Sera
2. Cell Panel
3. Family Studies
4. Blood Typing Procedures
5. Methods of Analysis

Results

1. Accuracy of Antigen Assignment
2. Specificities Present
3. Independence of Specificities
4. Gene Frequencies

Discussion

Introduction

The aim of this chapter is to determine the genetic relationships between the seventeen lymphocyte antigens detected in Chapter 5. Genetic relationships between antigens can be detected at two levels. Analysis can be made at the population level or by studying inheritance in families. Analysis at the population level is often carried out. Population material is usually easily obtained whereas appropriate families are not always available.

Following Bodmer et al. (1969), who quotes Mi and Morton (1966), there are six possible relationships between a pair of antigens (a and b). The relationships were originally discussed in terms of gametic frequencies. For simplification, I discuss the effect on the phenotypes.

1. Identical

The two antigens are the same (a = b).

2. Subtypic

One antigen only occurs when the other is present. The second antigen can occur without the former, i.e. one antigen is included within the other. The alleles are ab and b.

3. Codominant

There is no null allele in this relationship. Although both antigens may be inherited together. There are three allelles, a, b and ab.

4. Complementary

This corresponds to a two allele system. There are no null alleles and no alleles carrying both specificites. There are two alleles a and b.

5. Segregant

This corresponds to a three allele system with one silent allele such as the human ABO blood group. No allele carries both antigens. There are three alleles a, b and - (blank).

6. Permuted

This is the general relationship expected between different antigens. The antigens may or may not be associated in the population. There are four possible alleles, a, b, ab and - (blank).

Identical and subtypic relationships are easily recognised from their phenotypic patterns. Codominant and complementary pairs both lack the null phenotype. They can be distinguished only by the existence of the gamete carrying both antigens. Segregant pairs can be recognised by the absence of the gamete carrying both antigens. Further, the segregant, codominant and complementary relationships are expected to have a negative correlation. The correlation is given by:-

$$r = \frac{ad - bc}{(a+b)(c+d)(a+c)(b+d)}$$

where/

where

a = number of individuals who possess both antigens

b = number of individuals who possess the first antigen only

c = number of individuals who possess only the second antigen

d = number of individuals who possess neither antigen

a + b + c + d = total number of individuals tested

In general, the majority of associations between white cell antigens fall into category six. To quote Bodmer et al. (1969) "A population association between two antigens, or, more generally, between two genetically determined traits, may be caused by one or more of the following factors:

(a) The traits may be multiple effects of the same gene. An interesting example of this is the association between red cell ABO compatibility and skin graft survival (Ceppellini et al., 1966; Dausset and Rapaport, 1966).

(b) The traits may be the result of epistatic interaction between two or more genes. A striking example is the interaction between the Lewis and secretor loci, such that Le^b is only found on the red cells of individuals with at least one of the dominant alleles at each locus (see Race and Sanger, 1962).

(c) There may be selective interactions between the loci. Probably the best example of this in man is the association between G6PD deficiency, thalassemia and resistance to malaria in certain areas of the Mediterranean (see e.g. Motulsky, 1964).

(d) Departures from random mating due to inbreeding, assortative mating or population stratification can lead to non-random associations/

associations between genes. The effects of inbreeding in human populations are likely to be quite small, and assortative mating with respect to cryptic genetic characters, such as blood group antigens, is unlikely. Population stratification on the other hand, particularly as a result of recent racial admixture, may be a very significant general source of non-random association between unlinked polymorphic loci. For example, in a random sample of the American population one should expect a negative association between the allele R_o (cDe) of the Rhesus system and the Duffy allele, Fy^a, and a positive association between R_o and the Kidd allele, Jk^a. This is because R_o and Jk^a occur with a relatively higher frequency in African, than in Caucasian populations, while Fy^a has a much higher frequency in Caucasians than Africans. The magnitude of the associations found in the American sample will depend on the proportion of individuals with African ancestry in the sample. The associations will, presumably, eventually disappear if random mating with respect to racial ancestry is established on a more or less permanent basis.

(e) The last, and from our point of view most important, cause of non-random association between genes is allelism or very close linkage. The negative association between the antigens A and B of the ABO system was, of course, the basis for Bernstein's interpretation of the system in terms of three alleles and the association between the antigens S,s and M,N was the basis for assigning all these antigens to one system, consisting of the four 'alleles', MS, Ms, NS, Ns.

It is this last cause of genetic association which has been emphasised especially by red cell and white cell groupers. This is because the other causes can, mostly, be readily recognised or controlled and are in any case unlikely to have small effects relative to that resulting from the very close linkage between genes controlling antigens which are part of the same system."

Therefore if there is a significant association between two antigens, it has generally been taken to mean either very close linkage or allelism. In dogs, (Vriesendorp et al., 1977), chimpanzees (Balner et al., 1971a) and rhesus monkeys (Balner et al., 1971b) investigators have tried to distinguish between allelism and independence by applying two chi-square tests. The chi-square for allelism and the chi-square for independence (Andresen et al., 1963; Andresen and Baker, 1964). The chi-square for allelism is calculated thus -

a) the gene frequencies in the sample are first calculated,

b) the expected values for each class in the 2 x 2 table are calculated (i.e. ++, +-, -+, --). This procedure assumes Hardy Weinberg equilibrium. The expected value of the ++ class is given by 2pq.N. The expected values of the other combinations can be computed directly or more simply by subtraction since the marginal totals will be the same as for the observed frequencies

c) the chi-square for allelism is calculated by summing over all four entries the value:-

$$\text{Chi-square} = \frac{(\text{observed} - \text{expected})^2}{\text{expected}}$$

A significant deviation from expected indicates that the genes controlling the two antigens are not in Hardy-Weinberg equilibrium.

The chi-square for independence is calculated similarly except that the expected values are calculated assuming independence of the two genes (i.e. they are unlinked or if linked, then they are in linkage equilibrium). Thus, the expected frequency of animals positive for both traits is given by $(a+b)(a+c)/n$ where a, b and c are as before and n = total number of individuals tested.

Andresen et al. (1963) state that the hypothesis (i.e. allelism or independence) which gives the smallest chi-square is the most likely to be true and should therefore be tentatively accepted. Andresen and Baker (1964) point out that this method cannot be used to test (or exclude) the hypothesis that the two sera detect four alleles at one locus. (The four alleles are a null allele, an allele which carries one antigen, an allele which carries the other antigen and an allele which carries both antigens). The authors also point out that the conclusions (i.e. allelism or independence) should be tested by family studies.

I have not tried an analysis at the population level of my sheep data. This is for three reasons. Firstly, the majority of my sheep come from the Damline breed. This breed has been recently produced from four breeds. This recent mixing of different breeds is likely to lead to non-random associations between polymorphic loci. Secondly, studies in other species have shown that linkage equilibrium is not usually the case between the alleles of different genes in the MHC. Linkage disequilibrium might therefore be expected in sheep.

Finally,/

Finally, sheep are usually kept in small flocks and a relatively small number of rams are used in each generation. This type of husbandry means that inbreeding is likely to be quite significant. Inbreeding will also cause non-random association between different genes.

Instead, I have looked at the inheritance of lymphocyte antigens in sheep families. I have used the information from family studies to produce an hypothesis of genetic control.

Results

The inheritance of seventeen lymphocyte antigens in eleven ram families is given in Tables 1 to 11. The family material presented here is the same as that utilised in Chapter 5. The phenotype of each animal has been listed. For each lamb, I have listed the known parental contribution. On the assumption that only one genetic system was involved, I then assigned genotypes to all sheep. Certain specificities could not be assigned to genotypes. They have been listed separately. As noted in Chapter 5, several lambs were positive for antigens that both parents lacked. Genotypes have not been assigned to these sheep. The specificities which show anomalous inheritance have been bracketed.

Each ram family is discussed separately.

Family 1 (Table 1) is a Finnish Landrace family from Moredun. The ram is apparently homozygous for SP9. I have not been able to assign SP9 to either haplotype in animals 004, 005 or 008.

Table 1

Key:

*	homozygosity cannot be excluded
!	Antigen not assigned in maternal haplotype only
R	Ram
E	Ewe
O	Offspring

TABLE 1

FAMILY 1

ANIMAL	STATUS	PHENOTYPE	PARENTAL CONTRIBUTION	POSSIBLE GENOTYPE	UNASSIGNED ANTIGENS
034 K889	R	9		9/9	
001 A232	E	2,6		2/6	
011 507	01	6,9	9/6	9/6	
002 C356	E	2,6		2/6	
012 505	01	2,9	9/2	9/2	
003 F922	E	2,6		2/6	
010 502	01	2,9	9/2	9/2	
004 3Z31	E	3A,6,9		6/3A	9
013 503	01	6,9	NK/6	9/6	9!
005 C303	E	3A,9,10		-/3A 10	9
018 514	01	9	NK/NK	9/-	9!
006 F944	E	9		9/*	
014 509	01	9	NK	9/9	
015 510	02	9	NK	9/9	
007 C354	E	2,6,10		2/6 10	
019 516	01	2,9	9/2	9/2	
020 517	02	2,9	9/2	9/2	
008 3Z37	E	6,9		6/-	9
016 512	01	9	NK/NK	9/-	9!
017 513	02	6,9	NK/6	9/6	9!

Family 2 (Table 2) is also from Moredun. The ram is a Dorset Horn while the mother is a Finnish Landrace. The father appears to pass on SP5, SP7, SP8, SP9 and SP10 to the lamb. Antigen SP6 cannot be assigned to genotypes. Animal 032 (not shown) is the full-sib of ram 031. Animal 032 is positive for SP5, SP7, SP8, SP9 and SP10, thus supporting the assumption that SP5, SP7, SP8, SP9 and SP10 are on the same haplotype.

Family 3 (Table 3) is an East Friesland family from Skedsbush. The East Friesland breed originates in the Netherlands. Specificity SP9 could not be assigned as all three sheep are SP9 positive.

As with Family 3, Family 4 (Table 4) were East Frieslands from Skedsbush. The genotype of animal 251 could not be determined. The SP2 antigens could have come from either parent.

Families 5, 6, 7, 8 and 9 are all Damlines from Skedsbush. In Family 5 (Table 5) there is one instance of anomalous antigen inheritance. Animal 163 shows an anomalous inheritance of two antigens, SP3A and SP9. As both specificities are well defined and as two specificities are involved, the result is unlikely to be due to false antigen assignment. The parentage of animal 163 appears to be incorrect and therefore a genotype has not been assigned to it.

The remaining animals in family 5 show no anomalies. The observed inheritance is in agreement with that expected if all specificities are controlled by a single genetic system. Antigens 2 and 6 behave as alleles in the ram. All offspring receive either SP2 or SP6.

TABLE 2

FAMILY 2

ANIMAL	STATUS	PHENOTYPE	PARENTAL CONTRIBUTION	POSSIBLE GENOTYPE	UNASSIGNED ANTIGENS
031 N584	R	5,6,7,8,9,10,15		5 7 8 9 10/15	6
030 K910	E	2,6		2/*	6
029 PT562	01	2,5,6,7,8,9,10	5 7 8 9 10/2	5 7 8 9 10/2	6

TABLE 3

FAMILY 3

ANIMAL	STATUS	PHENOTYPE	PARENTAL CONTRIBUTION	POSSIBLE GENOTYPE	UNASSIGNED ANTIGENS
023 LO97	R	9,14		14/-	9
252 8J198	E	4A,5,9		4A/5	9
253 9J225	O1	4A,9,14	14/4A	14/4A	9

TABLE 4

FAMILY 4

ANIMAL	STATUS	PHENOTYPE	PARENTAL CONTRIBUTION	POSSIBLE GENOTYPE	UNASSIGNED ANTIGENS
027 7J398	R	9		9/*	
247 6J343	E	8,9		8/-	9
248 9JO13	O1	8,9	NK/8	9/8	9!
250 7JO21	E	5,9		5/-	9
251 9J11O	O1	9	NK/NK	9/-	9!

TABLE 5

FAMILY 5

ANIMAL	STATUS	PHENOTYPE	PARENTAL CONTRIBUTION	POSSIBLE GENOTYPE	UNASSIGNED ANTIGENS
026 8J100	R	2,6		2/6	
067 7J205	E	5,9,12		5 9/12	
068 9J042	01	2,5,9	2/5 9	2/5 9	
069 9J043	02	2,12	2/12	2/12	
085 8J038	E	3A,3B,9,10		3A 3B 9 10/*	
241 9J151	01	2,3A,3B,9,10	2/3A 3B 9 10	2/3A 3B 9 10	
094 6J320	E	2,3A,9		3A 9/3A	2
095 9J212	01	2,3A,9	NK/3A 9	2/3A 9	2!
096 9J213	02	2,3A,6	6/3A	6/3A	2!
097 9J214	03	2,3A,9	NK/3A 9	2/3A 9	2!
102 5J064	E	2,9,10		9 10/-	2
103 9J240	01	2	NK/NK	2/- -	2!
104 9J241	02	6,9,10	6/9 10	6/9 10	
105 9J242	03	6,9,10	6/9 10	6/9 10	
110 6J136	E	1,4B,9		1/4B 9	
111 9J293	01	4B,6,9	6/4B 9	6/4B 9	
112 9J294	02	4B,6,9	6/4B 9	6/4B 9	
116 6J358	E	1,3A,5,9,10		1 5 10/3A 9	
117 9J305	01	2,3A,9	2/3A 9	2/3A 9	
120 6J057	E	4B,5,6,9		4B 9/5	6
121 9J351	01	5,6	NK/5	6/5	6!
122 9J352	02	5,6	NK/5	6/5	6!
162 8J289	E	NULL		-/-	
163 9J526	01	2,(3A),(9)	NK/NK	UNASSIGNED	
164 9J527	02	6	6/-	6/-	
060 8J309	E	3A,4A,9		3A/4A 9	
059 9J575	01	4A,6,9	6/4A 9	6/4A 9	
.69 6J366	E	3A,9		3A 9/*	
.70 9J550	01	2,3A,9	2/3A 9	2/3A 9	

In Family 6 (Table 6), SP9 has not been assigned to genotypes as this specificity was present in the ram and seven out of eight ewes. Two lambs, animals 108 and 109, show anomalous antigen inheritance. As the antigens involved are well defined, the anomalous inheritance is probably due to a parentage error. For animal 109 this explanation is supported by the observation that two specificities are involved. Also, with the exception of animal 108, all the postulated offspring of ram 024 inherit either SP4B or SP10. This observation also supports the assumption of parentage error Animals 108 and 109 have been excluded from the subsequent analysis.

Five of the eight ewes are also positive for SP10. Therefore the inheritance of SP10 cannot always be unambiguously determined. Nonetheless, the ram (animal 024) appears to pass on either SP4B or SP10 to his offspring. Tentatively, SP4B and SP10 can be described as alleles.

In Family 7 (Table 7), all lambs with two exceptions appear to inherit either SP2 or SP9 and SP10 from the sire. SP9 and SP10 are inherited together on the same haplotype. The two exceptions are animals 127 and 128. Additionally, the two animals both show an anomalous inheritance of SP3A. The most likely explanation to account for the sire inheritance and the anomalous antigen inheritance is parentage error. As SP9 and SP10 are inherited together, they must be i) products of the same gene, or ii) products of closely linked genes, or iii) a serological artefact (i.e. different sera have been used to detect one specificity).

TABLE 6

FAMILY 6

ANIMAL	STATUS	PHENOTYPE	PARENTAL CONTRIBUTION	POSSIBLE GENOTYPE	UNASSIGNED ANTIGENS
024 8J202	R	4B,9,10		4B/10	9
070 7J011	E	2,9,10		2/10	9
071 9J070	01	4B,9,10	4B/NK	4B/10	9
072 9J071	02	9,10	NK/NK	10/10	9
073 9J072	03	9,10	NK/NK	10/10	9
074 5J013	E	2,3A,9		2/3A	9
075 9J079	01	3A,4B,9	4B/3A	4B/3A	9
076 9J080	02	2,9,10	10/2	10/2	9
240 9J081	03	2,9,10	10/2	10/2	9
077 5J118	E	2,9,10		2/10	9
078 9J120	01	4B,9,10	4B/NK	4B/10	9
079 9J121	02	9,10	NK/NK	10/10	9
080 9J122	03	9,10	NK/NK	10/10	9
106 7J450	E	2,4A,5,9		2/4A5	9
107 9J270	01	2,4B,9	4B/2	4B/2	9
108 9J271	02	2,(3A),9	UNASSIGNED	UNASSIGNED	
109 9J272	03	(3A),(3B),9,10	UNASSIGNED	UNASSIGNED	
141 8J221	E	3A,9,10		3A/10	9
142 9J434	01	3A,4B,9	4B/3A	4B/3A	9
157 7J537	E	2		2/*	
158 9J498	01	2,9,10	9 10/2	9 10/2	
159 9J499	02	2,9,10	9 10/2	9 10/2	
160 8J402	E	5,9,10		5/10	9
161 9J512	01	4B,9,10	4B/NK	4B/10	9
166 8J536	E	3A,9,10		3A/10	9
167 9J536	01	3A,9,10	NK/3A	10/3A	9
168 9J537	02	4B,9,10	4B/NK	4B/10	9

TABLE 7

FAMILY 7

ANIMAL	STATUS	PHENOTYPE	PARENTAL CONTRIBUTION	POSSIBLE GENOTYPE	UNASSIGNED ANTIGENS
022 8J227	R	2,9,10		2/9 10	
081 8J452	E	2,3A,9		2/3A 9	
084 9J131	01	2	NK/NK	2/2	
113 8J107	E	5,6,12		5 6/12	
114 9J303	01	2,5,6	2/5 6	2/5 6	
115 9J304	02	9,10,12	9 10/12	9 10/12	
126 6J292	E	2,9,11		UNASSIGNED	
127 9J371	01	(3A),9,11	UNASSIGNED	UNASSIGNED	
128 9J372	02	(3A),9	UNASSIGNED	UNASSIGNED	
129 8J043	E	2,4A,9,10		2/4A 9 10	
130 9J376	01	2	NK/NK	2/2	
143 7J100	E	4B,9		4B 9/*	
144 9J455	01	2,4B,9	2/4B	2/4B 9	
145 9J456	02	4B,9,10	10/4B	9 10/4B 9	
146 9J457	03	2,4B,9	2/4B	2/4B 9	
164 8J492	E	5,9,10,15		5/9 10 15	
165 9J582	01	2,9,10,15	2/15	2/9 10 15	

Possibilities i) and iii) are plausible as SP9 and SP10 show no association with each other in the population. Although, it could be that at least two loci are involved in the control of sheep lymphocyte antigens and also that the two loci are closely linked.

In Family 8 (Table 8), only the SP2 specificity can be detected on the ram's lymphocytes. There are six ewes with a total of ten offspring. Three of the ewes also have the SP2 specificity. The other three ewes who lack the SP2 specificity have a total of five lambs. All the five lambs inherit the SP2 specificity from the sire. The other five lambs also have the SP2 specificity, although this could have come from the mother. The most likely explanation for the two observations, that only SP2 can be detected on the ram and that all lambs inherit the SP2 specificity, is that the ram is homozygous for SP2.

In Family 9 (Table 9), the sire and seven out of eight ewes, possess the SP9 specificity. The one ewe (animal 171) which is negative for SP9 has two lambs. Both lambs inherit SP3A and SP9 from the ram. The two lambs suggest that SP3A and SP9 are inherited together. Alternatively, the antisera to SP9 may all be polyspecific and contain SP3A specificity. However, there is no evidence regarding the inheritance of SP4B and SP9. Therefore SP9 has not been assigned to genotypes.

I have not assigned genotypes to two lambs, animals 091 and 092. This is for two reasons. Firstly, both animals are positive for SP2/

TABLE 8

FAMILY 8

ANIMAL	STATUS	PHENOTYPE	PARENTAL CONTRIBUTION	POSSIBLE GENOTYPE	UNASSIGNED ANTIGENS
021 8J293	R	2		2/2	
086 7JO91	E	2,3A,9		3A 9/-	2
087 9J185	O1	2,3A,9	NK/3A 9	2/3A 9	2!
088 9J186	O2	2,3A,9	NK/3A 9	2/3A 9	2!
089 9J187	O3	2	NK/NK	2/-	2!
118 8JO25	E	3A,9,1O		3A/9 1O	
119 9J349	O1	2,9,1O	2/9 1O	2/9 1O	
136 6J261	E	2,4A,9		4A 9/-	2
137 9J411	O1	2	NK/NK	2/-	2!
138 8JO35	E	2,3A,9,1O		3A 9/1O	2
139 9J414	O1	2,1O	NK/NK	2/1O	2!
147 7J179	E	3A,9,1O		3A 9/9 1O	
148 9J466	O1	2,3A,9	2/3A 9	2/3A 9	
149 9J467	O2	2,9,1O	2/9 1O	2/9 1O	
154 7J464	E	4B,9,1O		4B 9/9 1O	
155 9J484	O1	2,9,1O	2/9 1O	2/9 1O	
156 9J485	O2	2,4B,9	2/4B 9	2/4B 9	

TABLE 9

FAMILY 9

ANIMAL	STATUS	PHENOTYPE	PARENTAL CONTRIBUTION	POSSIBLE GENOTYPE	UNASSIGNED ANTIGENS
025 8J459	R	3A,4B,9		3A/4B	9
090 5J039	E	1,4B,9		1/*	4B,9
091 9J191	01	(2)	UNASSIGNED	UNASSIGNED	
092 9J192	02	(2)	UNASSIGNED	UNASSIGNED	
093 9J194	03	1,4B,9	NK/NK	4B/1	4B!,9
098 6J133	E	3A,3B,9,10,15		3A 3B/10 15	9
099 9J222	01	4B,9,10,15	4B/10 15	4B/10 15	9
100 9J223	02	3B,4B,9,10	4B/3B 10	4B/3B 10	9
101 9J224	03	3A,9,10,15	NK/10 15	3A/10 15	9
123 7J399	E	3A,5,6,9		5 6/-	3A,9
124 9J365	01	3A,5,6,9	NK/5 6	3A/5 6	3A!,9
125 9J366	02	3A,9	NK/NK	3A/-	3A!,9
131 8J349	E	9,10		10/*	9
132 9J388	01	3A,9,10	3A/10	3A/10	9
133 7J161	E	3A,9,10		10/-	3A,9
134 9J400	01	3A,9	NK/NK	3A/-	3A!9
135 9J401	02	3A,9,10	NK/10	3A/10	3A!,9
150 6J234	E	3B,9,10		3B 10/-	9
151 9J481	01	3A,9	3A/NK	3A/-	9
152 9J482	02	3B,4B,9,10	4B/3B 10	4B/3B 10	9
153 9J483	03	3A,9	3A/NK	3A/-	9
171 7J345	E	2,8		2/8	
172 9J559	01	2,3A,9	3A 9/2	3A 9/2	
173 9J560	02	3A,8,9	3A 9/8	3A 9/8	
061 8J530	E	9		9/*	
062 9J576	01	4B,9	4B/NK	4B/NK	9
063 9J577	02	4B,9	4B/NK	4B/NK	9

SP2, although their parents are negative. Secondly, all lambs with the exception of these two, inherit either SP3A or SP4B from the sire. The two observations argue in favour of false parentage. Once animals 091 and 092 have been excluded, sixteen lambs remain. The paternal inheritance can only be conclusively determined for thirteen/sixteen lambs. For three lambs, the lamb and the ewe share a specificity with the ram. The other thirteen lambs inherit either SP3A or SP4B from the ram. Therefore SP3A and SP4B appear to be allelic.

Three lambs, animals 099, 100 and 101, show an interesting inheritance from the mother. The ewe is positive for SP3A, SP3B, SP10 and SP15. Animals 099 and 101 inherit SP10 and SP15 from the mother. They inherit SP4B and SP3A respectively from the sire. Animal 100 inherits SP4B from the sire and SP3B and SP10 from the mother. There are two alternative explanations for the observed maternal inheritance. Firstly, the genes controlling the four specificities are not linked. This is unlikely as, as in the other families, the lymphocyte antigens behave as part of a single genetic system. Secondly, the genes may be linked, in which case, recombination must have occurred. The most plausible explanation is one which requires the least number of cross-over events. If we postulate that SP3A and SP3B are on one haplotype and SP10 and SP15 are on the other, then only one cross-over is necessary to explain the maternal inheritance. This is schematically represented below:-

$$\frac{10 \quad 15}{\quad} \; P_1 \qquad\qquad \frac{3A \quad 15}{\quad} \; R_1$$

$$\frac{\quad}{3A \quad 3B} \; P_2 \qquad\qquad \frac{\quad}{10 \quad 3B} \; R_2$$

Animals 99 and 101 inherit the parent haplotype P_1. Animal 100 inherits the recombinant haplotype R_1. This probable case of recombination implies that at least two linked loci are involved in the genetic control of sheep lymphocyte antigens. Although, intragenic recombination cannot be excluded. No estimate can be made of the recombination frequency. Many cross-over events could have taken place but they would remain undetected. Cross-overs would not for example be detected in a) single offspring families; b) in homozygous parents; c) where an undetected antigen was involved, or d) where parents had specificities in common.

In Family 10 (Table 10) all animals possess specificities SP9 and SP13. Therefore these two specificities cannot be reliably assigned to genotypes. The maternal inheritance appears straightforward.

In Family 11 (Table 11), the sire possesses specificities SP9 and SP11. All four ewes also possess SP9. Therefore, this specificity cannot be reliably assigned to families. Specificity SP11 is inherited by all five lambs. Therefore the ram is probably homozygous for SP11.

The family data provided no evidence to contradict the assumption that only one genetic system is involved. Therefore it has been tentatively concluded that a single genetic system controls the inheritance of the seventeen sheep lymphocyte antigens detected in this study.

I am not able on the basis of this data to decide how many different loci control the antigens. There is only one example of possible recombination in the ninety-seven lambs. However, recombination could have occurred without being detected in several offspring. The highest number/

TABLE 10

FAMILY 10

ANIMAL	STATUS	PHENOTYPE	PARENTAL CONTRIBUTION	POSSIBLE GENOTYPE	UNASSIGNED ANTIGENS
222 7Z001	R	9,13		-/-	9,13
177 4Z14	E	5,9,13		5/-	9,13
178 9FF5	01	9,13	NK/NK	-/-	9,13
180 5Z004	E	7,9,10,13	NK/7 10	-/7 10	9,13
181 9FF04	01	7,9,10,13	NK/7 10	-/7 10	9,13
182 5Z008	E	4A,9,13		4A/-	9,13
183 9FF01	01	4A,9,13	NK/4A	-/4A	9,13

TABLE 11

FAMILY 11

ANIMAL	STATUS	PHENOTYPE	PARENTAL CONTRIBUTION	POSSIBLE GENOTYPE	UNASSIGNED ANTIGENS
224 BLWEL	R	9,11		11/*	9
225 D667	E	4A,9		4A 9/-	
226 LO27	O1	11	11/-	11/-	
227 D5O6	E	4A,9		4A/*	9
228 9LL4O	O1	4A,9,11	11/4A	11/4A	9
229 D677	E	4A,9		4A/*	9
23O 9LL41	O1	4A,9,11	11/4A	11/4A	9
231 D674	E	4A,9		4A 9/-	
232 9LL46	O1	11	11/-	11/-	
233 2U151	E	8,9		8 9/-	
234 9LL57	O1	11	11/-	11/-	

number of antigens inherited on one haplotype is five (ram 031 and lamb 029). Animal 032 is the full-sibling of ram 031. 032 also possesses the five specificities present in 031 and 029. This supports the conclusion that the five specificities are on one haplotype. However, the sera could be oligospecific and the same specificity might be detected by different clusters. Several animals appear to pass on three antigens to their offspring (e.g. ewe 064 passes on SP9, SP10 and SP15 to her lamb, animal 065). The distribution of specificities amongst the sheep is given in figure 1. The highest number of antigens possessed by any one animal is seven (animals 029 and 031).

Discussion

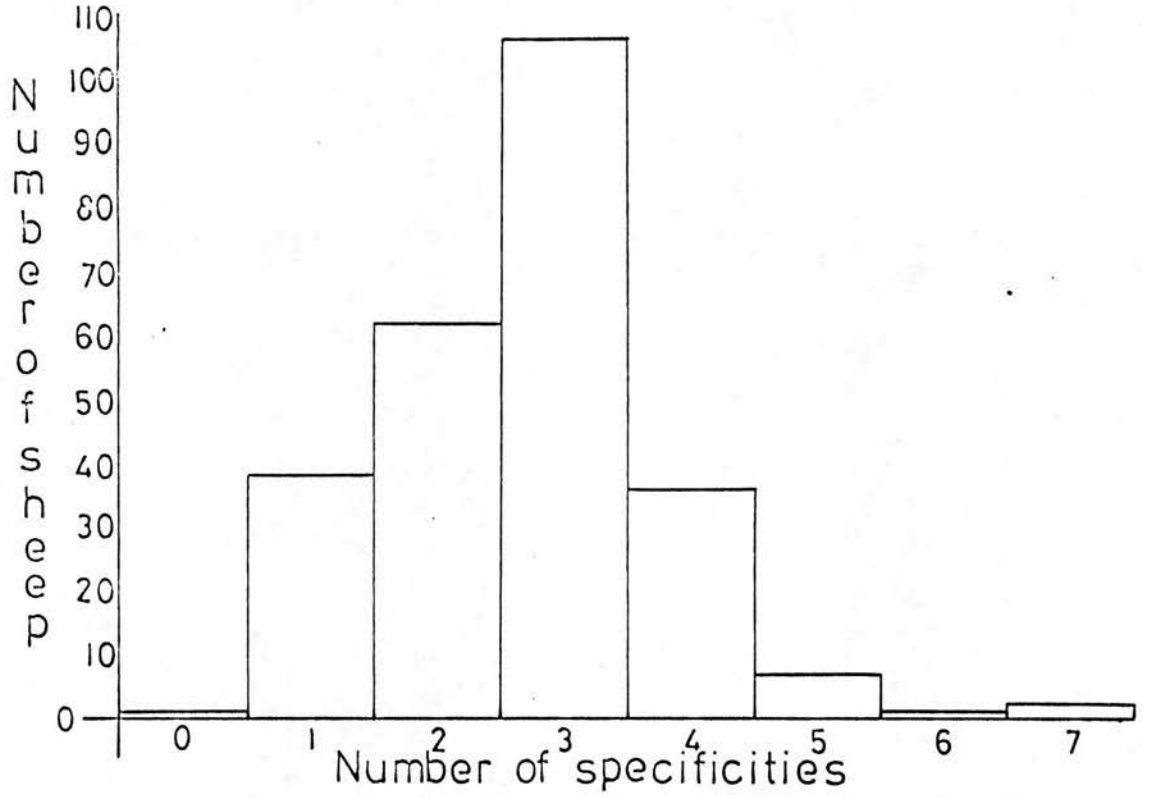
There are four possible explanations for the observed distribution of antigens.

1) Different groups of sera are in fact detecting the same antigen. This is unlikely to account fully for the observed results. Different groups of antigens are involved in the haplotypes with several antigens. Also, with the exception of SP9, there were no significant positive associations between different antigens (see previous chapter). However, the absorptions necessary to formally exclude this possibility have not been done.

2) Several of the reactions in the sheep with many specificities are due to false positives. This is also unlikely as the specificities are inherited in families. However this possibility cannot be entirely excluded.

Figure 1

Distribution of 17 Lymphocyte Antigens Amongst 253 Sheep



3) The specificities are all coded for by different alleles of the same locus. In this hypothesis, one allele would carry (say) antigens SP9 and SP3A, another would carry antigens (say) SP9 and SP10. If this were the case, several antigens would be expected to be included within other antigens. This may be the case for SP9 and its included specificities. This does not appear to be the case for the other specificities. It is possible that the different antigens on the same molecule need not show sub-group relationships. This possibility cannot be ruled out without further study.

4) The fourth possibility is that the different antigens on the same haplotype are coded for by different loci. This is the result expected from work in other species. Under this hypothesis it is still possible that more than one antigen would be present on one molecule. The supposition that at least two loci are involved is given some weight by the observation of recombination in one family. However, intragenic recombination is not unknown (Whitehouse, 1965). If we exclude SP9 as it may not be an independent antigen, the highest number of antigens on one haplotype is four. Four antigens have also been observed on one haplotype in humans (Goodfellow and Payne, 1978).

In summary, this chapter has shown that one genetic system controls the inheritance of lymphocyte antigens in sheep. This result is in agreement with results obtained in other species (Götze, 1977). Antisera from a variety of sheep breeds and a number of laboratories have been used to reach this conclusion.

Millot (1978) has reported that at least three genetic systems are involved in the control of sheep lymphocyte antigens. My results provide no support for this observation.

The structure of the gene system controlling lymphocyte antigens is not yet known. At least two genes appear to be involved. There is no evidence for more than four loci.

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REFERENCES

C.J.Abeyounis & F.Milgrom 1969.
Tissue isoantigens shared by rabbits and mice.
Transplant.Proc. 1 556-559.

C.J.Abeyounis,F.Milgrom & E.Witebsky 1964.
Homotransplantion antibodies detected by mixed agglution with cell culture monolayers.
Nature 203 313-314.

T.E.Adams,M.R.Brandon & B.Morris 1979.
The potential of the I region of the bovine major histocompatibility complex.
Anim.Blood Grps.Biochem.Genet.10 155-163.

S.Adler 1948.
Origin of the golden hamster Cricetus auratus as a laboratory animal.
Nature 162 256-257.

S.Ahrons 1971.
HLA antibodies: Influence on the human fetus.
Tissue Antigens 1 122-128.

S.Ahrons & F.Kissmeyer-Nielson 1973.
HL-A typing: Influence of methodological variation.
Symp.Ser.Immunobiol.Std. 18 85-88.

E.Albert & D.Götze 1977.
The major histocompatibility system in man.
The Major Histocompatibility System in Man and Animals. D.Götze (ed) Springer-Verlag Berlin Heidelberg.pp7-77.

E.Albert,K.Kano,C.J.Abeyounis & F.Milgrom 1969.
Detection of human lymphocyte isoantigens by rabbit homotransplantation sera.
Transplantation 8 466-471.

E.D.Albert,M.R.Mickey & P.I.Terasaki 1973.
A new approach to cross-reactivity in the HL-A system.
Symp.Ser.Immunobiol.Std. 18 156-164.

E.D.Albert,S.Scholz,J.Bertrams,R.W.Ewald,S.Seidl, W.Ratschko & E.Westphal 1975.
Representative HL-A frequencies of several German populations.
Z.Immunitatsf. 148 367-371.

D.Albrecsten,B.G.Solheim & E.Thorsby 1977.
Stimulating capacity of human lymphoid cell subpopulations in mixed lymphocyte cultures.

Scand.J.Immunol. 7 427-434.

L.Alderson 1978.
The Chance to Survive.Rare Breeds in a Changing World.
Cameron & Tayleur (Books) Ltd. London

F.H.Allen 1974.
Linkage of HL-A and GBG.
Vox Sang. 27 382-384.

J.P.Allison,L.E.Walker,W.A.Russell,S.Ferrone R.A.Reisfield,M.A.Pellegrino,J.A.Frelinger & J. A.Silver 1978.
Structural analysis of murine and human Ia alloantigens (abstr).
J.Immunol. 121 1610.

C.Alper 1978.
Personal communication.
Cited by Lachmann and Hobart 1978 op.cit.

B.J.Alter,D.J.Schendel,M.L.Bach,F.H.Bach,J.Klein & J.H.Stimpfling 1973.
Cell mediated lympholysis:Importance of serologically defined H-2 antigens.
J.Exp.Med. 137 1303-1313.

B.Altman & M.Simonsen 1964.
Cytotoxic antibody and hemagglutinin in canine homotransplantation.
Ann.N.Y.Acad.Sci. 120 28-34.

J.D'Amaro 1970.
The micro-complement fixation test.1.A solution of the technical problems of HL-A typing?
Vox Sang. 18 334-341.

B.Amorena & W.H.Stone 1978.
Serologically defined (SD) locus in cattle.
Science 201 159-160.

D.B.Amos 1953.
The agglutination of mouse leucocytes by isoimmune sera.
Brit.J.Exp.Pathol. 34 464-470.

D.B.Amos 1976.
Freezing and thawing lymphocytes.
NIAID Manual of Tissue Typing Techniques.
J.G.Ray,D.B.Hare,P.D.Pedersen & D.I.Mullally (eds).
DHEW Publication No. (NIH) 78-545 U.S.A.pp186-187.

D.B.Amos & F.E.Ward 1975.
Immunogenetics of the HL-A system.
Physiol.Rev. 55 206-246.

E.Andresen & L.N.Baker 1964.
Distinguishing between various genetic theories using random population material.
Immunogenet.Letter 3 95-99.

E.Andresen,L.N.Baker & K.E.Rowe 1963.
The usefulness and limitation of two-by-two tables in distinguishing between allelism and non-allelism based on random population material.
Immunogenet.Letter 3 15-20.

H.Anudi,L.Rask,L.Ostberg & P.A.Petersen 1975.
The subunit structure of thymus leukemia antigens.
Biochem. 14 5046-5054.

A.W.M.Appelman & H.Balner 1972.
Mixed leucocyte cultures in rhesus monkeys.
Transplant.Proc. 4 17-20.

A.Arnaiz-Villena,P.Halloran & C.S.David 1975.
Specific inhibition of lymphoid complement receptors by anti-H-2 sera:Evidence for a new H-2 linked polymorphism.
J.Immunogenetics 2 415-425.

J.Ayres & P.Cresswell 1976.
HLA-B specificities and w4,w6 specificities are on the same polypeptide.
Eur.J.Immunol. 6 794-799.

F.H.Bach,R.J.Albertini,D.B.Amos,R.Ceppellini, P.L.Mattiuz & V.C.Miggiano 1969.
Mixed leucocyte culture studies families with known HL-A genotypes.
Transplant. Proc. 1 339-341.

F.Bach,M.Bach & P.Sondel 1976.
Differential function of major histocompatibility complex antigens in T lymphocyte activation.
Nature 259 273-281.

F.H.Bach & J.J. van Rood 1976a.
The major histocompatibilty complex - genetics and biology (first of three parts).
New Engl.J.Med. 295 806-813.

F.H.Bach & J.J. van Rood 1976b.
The major histocompatibilty complex - genetics and biology (second of three parts).
New Engl.J.Med. 295 872-878.

F.H.Bach & J.J. van Rood 1976c.
The major histocompatibilty complex - genetics and biology (third of three parts).
New Engl.J.Med. 295 927-936.

L.D.Bacon,J.R.Kite & N.R.Rose 1973.
Immunogenetic detection of B locus genotypes in chickens with autoimmune thyroiditis.
Transplantation 16 591-598.

K.Bagshaw & S.Lawler 1975.
The immunogenicity of the placenta and trophoblast.
Immunobiology of Trophoblast. R.G.Johnson, C.W.S.Howe & M.H.Johnson (eds) Cambridge University Press pp171-191.

E.Bailey,C.Stormont,Y.Suzuki & A Trommershausen-Smith 1979.
Linkage of loci controlling alloantigens on red blood cells and lymphocytes in the horse.
Science 204 1317-1319.

C.M.A.Baker & C.Manwell 1977.
Heterozygosity of the sheep:Polymorphism of 'malic enzyme',isocitrate dehydrogenase (NADP+), catalase and esterase.

Austr.J.Biol.Sci. 30 127-140.

J.Balcarová,J.Derka,K.Hála & T.Hraba 1974.
Genetic control of immune response to the dinitrophenol group in inbred lines of chickens.
Folia Biol. 20 346-349.

H.Balner 1973.
Current knowledge of the histocompatibility complex of rhesus monkeys (a brief review).
Transplant.Rev. 15 50-61.

H.Balner 1977.
The major histocompatibilty system of subhuman primate species.
The Major Histocompatibility System in Man and Animals. D.Gotze (ed) Springer-Verlag Berlin Heidelberg.pp79-127.

H.Balner,F.J.Cleton & J.G.Fernisse (eds) 1965a
Histocompatibility Testing 1965.
Munksgaard Copenhagen.

H.Balner,J.D'Amaro & T.P.Visser 1974a
Tissue typing of chimpanzees.1.Evidence for two allelic series of leukocyte antigens.
Transplant.Proc. 6 141-149.

H.Balner,H.Dersjant,A.van Leeuwen & J.J.van Rood 1967.
Identification of two major leukocyte antigens of rhesus monkeys and their relationship to histocompatibility.
Histocompatibility Testing 1967.E.S.Curtoni,P.L. Mattiuz & R.M.Tosi (eds) Munksgaard,Copenhagen.pp267-277.

H.Balner,H.Dersjant & J.J.van Rood 1965b.
A method to relate leukocyte antigens and transplantation antigens in monkeys.
Transplantation 3 230-234.

H.Balner,H.Dersjant & J.J.van Rood 1965c
Leukoagglutinating isoantibodies in rhesus monkeys.
Transplantation 3 402-422.

H.Balner,M.E.Dorf,M.L.De Groot & B.Benacerraf 1973a
The histocompatibility complex of rhesus monkeys.III. Evidence for a major MLR locus and histocompatibility linked Ir genes.
Transplant.Proc. 5 1555-1560.

H.Balner,B.W.Gabb,J.D'Amaro,W.van Vreeswijk & T.P.Visser 1974b
Evidence for two linked loci controlling the serologically defined leukocyte antigens of chimpanzees (ChL-A).
Tissue Antigens 4 313-328.

H.Balner,B.W.Gabb,H.Dersjant,W.van Vreeswijk, A.van Leeuwen & J.J.van Rood 1971a.
Heterologous antisera for human histocompatibility testing.
Transplant.Proc. 3 1088-1098.

H.Balner,B.W.Gabb,H.Dersjant,W.van Vreeswijk & J.J.van Rood 1971b.
Major histocompatibility locus of rhesus monkeys.
Nature New Biol. 230 177-180.

H.Balner,B.Gabb,E.Toth,H.Dersjant & W.van Vreeswijk 1973b

The histocompatibility complex of rhesus monkeys.1. Serology and genetics of the RhL-A system. Tissue Antigens 3 257-272.

H.Balner & W.van Vreeswijk 1975.
The major hisocompatibility complex of rhesus monkeys (RhL-A).V.Attempts at serological identification of MLR determinants and postulation of an I region in the RhL-A complex.
Transplant.Proc. 7 13-20.

H.Balner,W.van Vreeswijk & J.H.Roger 1976.
Ia-like antigens of Rhesus monkeys:Current state of serology and genetics.
Transplant.Rev. 30 3-17.

H.Balner,W.van Vreeswijk,J.H.Roger & J.D'Amaro 1978.
The major histocompatibility complex of chimpanzees: Identification of several new antigens controlled by the A and B loci of ChL-A.
Tissue Antigens 12 1-18.

C.J.Barnstable,E.A.Jones & M.J.Crumpton 1978.
Chemistry and genetics of HLA antigens.
Brit.Med.Bull. 34 241-246.

R.Barr & S.Perry 1976.
Lymphocyte purification from normal human peripheral blood by filtration through columns of cotton wool.
Scand.J.Haematol. 17 300-304.

J.R.Batchelor 1965.
Antibody response of humans to allogeneic skin grafts.
Histocompatibility Testing 1965 H.Balner,F.J. Cleton & J.G.Eernisse (eds)Munksgaard Copenhagen pp257-262.

B.Benacerraf 1972.
The cellular locus of action of histocompatibility linked Ir genes.
Genetic Control of Immune Responsiveness.
H.McDevitt & M.Landy (eds) Academic Press,New York.

B.Benacerraf 1974.
Immune response genes.
Scand.J.Immunol. 3 381-386.

B.Benacerraf 1977a.
Genetic control of specific immune responses.
Ann.Immunol. (Inst.Pasteur) 128C 385-392.

B.Benacerraf 1977b
Role of major histocompatibility complex in genetic regulation of immunologic responsiveness.
Transplant.Proc 9 825-831.

B.Benacerraf & D,Katz 1975a
The histocompatibility-linked immune response genes.
Adv.Cancer Res. 21 121-173.

B.Benacerraf & D.H.Katz 1975b.
The nature and function of histocompatibility-linked immune response genes.
Immunogenetics and Immunodeficiency B.Benacerraf (ed) Medical and Technical Publishing Co.,London. pp117-177.

B.Benacerraf & R.Germain 1978.
The immune response genes of the major histocompatibilty complex.
Immunol.Rev.38 70-119.

V.Benda 1971.
Identification of B antigen on chicken somatic cells.
Proc.VIIth.Internatl.Symp.Lab.Anim. Hruba Skala.
pp49-55.Cited by K.Hala 1977 op.cit.

K.Bender & K-H.Grzeschik 1976.
Assignment of the genes for human glyoxylase 1 to chromosome 6 and for human esterase D to chromosome 13.
Cytogenet.Cell Genet. 16 93-96.

A.Benedict,L.Pollard & P.Maurer 1977.
Genetic control of immune responses in chickens.II.
Response to methylated bovine serum albumin-poly (Glu^{60} Ala^{30} Tyr^{10})n aggregates.
Immunogenetics 4 199-204.

D.Bennet 1975.
The T-locus of the mouse.
Cell 6 441-454.

M.Berah,J.Hors & J.Dausset 1970.
A study of HL-A antigens in human organs.
Transplantation 9 185-192.

I.Bernier,A.Dautigny,J.Colombani & P.Jolles 1976.
Investigations on human leukocyte antigens (HLA) from urine.
FEBS Letters 63 320-322.

D.Bernoco,S.Cullen,G.Scudeller,G.Trinchieri & R.Ceppellini 1973.
HL-A molecules at the cell surface.
Histocompatibity Testing 1972 J.Dausset & J.Colombani (eds) Munksgaard,Copenhagen pp527-537.

M.J.Bevan 1975.
The major histocompatibilty complex determines susceptibilty to cytotoxic T cells directed against minor histocompatibilty antigens.
J.Exp.Med 142 1349-1364.

W.B.Bias & D.B.Marsh 1975.
HL-A linked antigen E immune response genes:
An improved hypothesis.
Science 188 375-377.

R.E.Billingham & G.H.Lampkin 1957.
Further studies in tissue homotransplantation cattle.
J.Embryol.Exp.Morphol. 5 351-357.

D.Bitter-Suermann,M.Kronke,T.Hoffman,A.F.Geczy & U.Hadding 1978.
Evidence for a linkage group of the polymorphic complement proteins C4,C2 and factor B related to the MHC of the guinea pig (GPLA) (abstr).
J.Immunol. 120 1765.

L.Black 1967.
Histocompatibilty in the rabbit.
Transplantation 5 390-409.

E.P.Blankenhorn,M.Cecka,D.Götze & L.Hood 1978.
Chemical characterisation of rat Ia antigens (abstr).
J.Immunol. 121 1610.

M.H.Blunt 1975.
Cellular elements of ovine blood.
The Blood of Sheep. Composition and Function
M.H.Blunt (ed) Springer-Verlag Berlin.

M.Bobrow,J.Bodmer,W.Bodmer,H.O.McDevitt,J.Lorber &
P.Swift 1975.
The search for a human equivalent of the
mouse t-locus-Negative results from a study of HL-A
types in spina bifida.
Tissue Antigens 5 234-237.

J.G.Bodmer 1973.
Unpublished observations.
Cited by Bagshawe and Lawler 1975 op.cit.

J.G.Bodmer 1978.
Ia antigens. Definition of the HLA-DRw specificities.
Brit.Med.Bull 34 233-240.

W.F.Bodmer 1972.
Evolutionary significance of the HL-A system.
Nature 237 139-145.

W.F.Bodmer 1973.
A new genetic model for allelism at histocompatibilty
and other complex loci:Polymorphism for control
of gene expression.
Transplant. Proc. 5 1471-1475.

W.F.Bodmer 1978.
The HLA system.
Brit.Med.Bull. 34 213-314.

W.F.Bodmer,J.R.Batchelor,J.G.Bodmer,H.Festenstein &
P.J.Morris (eds) 1978.
Histocompatibility Testing 1977.
Munksgaard,Copenhagen.

W.F.Bodmer,J.G.Bodmer,D.Ihde & S.Adler 1969.
Genetic and serological analysis of the HL-A
leukocyte system.
Computor Applications in Genetics N.E.Morton (ed)
Univ. Hawaii Honolulu pp117-128.

W.F.Bodmer & J.Felsenstein 1967.
Linkage and selection:Theoretical analysis of the
deterministic two locus random mating model.
Genetics 57 237-265.

W.F.Bodmer,E.A.Jones,D.Young,P.N.Goodfellow,J.G.Bodmer,
H.M.Dick & C.M.Steel 1975.
Serology of human Ia type antigens detected on
lymphoid lines: An analysis of the VI workshop sera.
Histocompatibilty Testing 1975 F.Kissmeyer-Nielsen
(ed) Munksgaard,Copenhagen pp677-684.

W.F.Bodmer & R.Payne 1965.
Theoretical cosideration of leukocyte grouping
using multispecific sera.
Histocompatibility Testing 1965 H.Balner,F.J.Cleton &
J.G.Eernisse (eds) Munksgaard,Copenhagen pp141-149.

W.Bodmer,M.Tripp & J.G.Bodmer 1967.
Application of a flourochromatic cytotoxic assay to human leukocyte typing.
Histocompatibility Testing 1967 E.S.Curtoni, P.L.Mattiuz & R.M.Tosi (eds) Munksgaard,Copenhagen pp341-350.

A.E.Bogden & P.M.Aptekman 1960.
The 'R-1' factor a histocompatibity antigen in the rat.
Cancer Res. 20 1272-1282.

M.Boroskowa & P.Demant 1967.
Specificity of cytotoxic antibodies in typing sera against blood group antigens.
Folia Biol. 13 473-475.

J.Bouw & A.Fiorentini 1970.
Structure of loci controlling complex blood group systems in cattle.
Proc.11th Eur.Conf.Animal Blood Grps.1968.
W.Junk,The Hague pp109-113.

B.A.Bradley & H.Festenstein 1978.
Cellular typing.
Brit.Med.Bull. 34 223-232.

A.Boyum 1968.
Separation of leukocytes from blood and bone marrow.
Scand.J.Clin.Invest.21 Suppl. 97 9-109.

G.J.Brewer & C.F.Sing 1969.
Survey of isozymes of human erythrocytes.
Biochemical Methods in Red Cell Genetics J.J.Yunis (ed) Academic Press, New York pp377-390.

W.E.Briles,C.P.Allen & T.W.Millen 1957.
The B blood group system of chickens.1.
Heterozygosity in closed populations.
Genetics 42 631-648.

R.Bull & H.Vriesendorp 1977.
Unpublished observations.
Cited by Vriesendorp et al.1977 op.cit.

G.W.Butcher & J.C.Howard 1977.
A recombinant in the major histocompatibility complex of the rat.
Nature 266 362-364.

A.E.Butterworth 1977.
The eosinophil and its role in immunity to helminth infection.
Current Topics Microbiol.Immunol. 77 127-168.

J.Caldwell C.F.Bryan,P.A.Cumberland & D.F.Weseli 1977.
Serologically detected lymphocyte antigens in Holstein cattle.
Anim.Blood Grps.Biochem.Genet. 8 197-207.

J.Caldwell & P.A.Cumberland 1978.
Cattle lymphocyte antigens.
Transplant.Proc. 10 889-892.

M.C.Carr,D.P.Stites & H.H.Fudenberg 1974.
Cellular immune aspects of the human fetal-maternal relationship.III.Mixed lymphocyte reactivity between related maternal and cord blood lymphocytes.

Cell.Immunol. 11 332-341.

L.L.Cavalli-Sforza & W.F.Bodmer 1971.
The Genetics of Human Populations.
W.H.Freeman,San Francisco.

C.K.Chai 1974.
Genetic studies of histocompatibility in rabbits:
Identification of major and minor genes.
Immunogenetics 1 126-132.

T.C.Chanh,A.Benedict & H.Ablanalp 1976.
Association of serum haemolytic complement levels
with the major histocompatibility complex in chickens.
J.Exp.Med.144 555-561.

P.Chardon,M.Vaiman,C.Renard & B.Arnoux 1977.
Presence of soluble SLA histocompatibility antigen
in pig plasma.
Anim.Blood Grps.Biochem.Genet. 8 139-148.

P.Chardon,M.Vaiman,C.Renard & B.Arnoux 1978.
Pig histocompatibility antigens and B-2microglobulin.
Transplantation 26 107-112.

R.N.Charlton & C.M.Zmijeski 1970.
Soluble HLA-A7 antigen:Localisation in the lipoprotein
fraction of human serum.
Science 170 636-637.

K.C.Cochrum,R.K.Main & S.L.Kountz 1971.
A new matching technique:the mixed skin cell-
leukocyte reaction (MSLR).
Surgery 70 97-102.

F.Cohen,W.Zuelzer,D.C.Gustafson & M.M.Evans 1964.
Mechanisms of immunisation.1.The transplacental
passage of fetal erythrocytes in homospecific pregnancies.
Blood 23 621-646.

W.M.Collins W.E.Briles,R.M.Zsigray,W.R.Dunlop,A.C.Corbett
K.K.Clark,J.L.Marks & T.P.McGrail 1977.
The B locus (MHC) in the chicken: Association with
the fate of RSV-induced tumours.
Immunogenetics 5 333-343.

Z.V.Collins,P.F.Arnold,F.Peeton,G.S.Smith & R.Walford 1973.
A naturally occuring monospecific anti-HL-A8.
isoantibody
Tissue Antigens 3 358-363.

J.Colombani,J.D'Amaro,B.Gabb,G.Smith & A.Svejgaard 1971.
International agreement on a microtechnique of
platelet complement fixation (Pl C Fix).
Transplant.Proc. 3 121-126.

M.Colombani,J.Colombani,H.Dastot,S.Mayer,M.M.Tongio &
J.Dausset 1969.
Définition de deux nouveaux antigènes du système HL-A:
Da 19 et Da 20.Reaction croisées entre les antigènes
Da 19,Da 20,HLA-A5 et Da 6.
Rev.Franç.Étude Clin. Biol. 14 995-1003.

J.Colombani,M.Colombani,A.Benajam & J.Dausset 1967.
Leucocyte and platelet antigens defined by platelet
complement fixation test (antigens 1,5,6,11 and 14).
Histocompatibility Testing 1967 E.S.Curtoni,P.L.

Mattiuz & R.M.Tosi (eds)Munksgaard,Copenhagen.pp413-417.

J.Colombani,M.Colombani & J.Dausset 1970.
Cross reactions in the HL-A system with special reference to the Da 6 cross-reacting group.Description of HL-A antigens Da 22,Da 23,Da 24 defined by platelet complement fixation.
Histocompatibility Testing 1970 P.I.Terasaki (ed) Munksgaard,Copenhagen.pp79-92.

R.Cook,J.D.Capra,E.S.Vitteta & J.W.Uhr 1978.
Structural characterisation of murine Ia alloantigens (abstr).
J.Immunol. 121 1611.

R.R.A.Coombs,A.M.Coombs & D.G.Ingram 1961.
The Serology of Conglutination and its Relation to Disease.
Blackwell Scientific Publications,Oxford.

D.W.Cooper,L.F.Bailey & O.Mayo 1967.
Population data for the transferrin variants in the Australian Merino.
Aust.J.Biol.Sci. 20 959-966.

S.E.Cullen,J.H.Freed & S.G.Nathenson 1976.
Structural and serological properties of murine Ia alloantigens.
Transplant.Rev. 30 236-270.

B.Curman,L.Ostberg,L.Sandberg,K.Malmheden-Eriksen, G.Stalenheim,L.Rask & P.A.Petersen 1975.
H-2 linked Ss protein is C4 component of complement.
Nature 258 243-245.

R.Curry,M.Dierich,M.Pellegrino & J.Hoch 1976.
Evidence for linkage between HLA antigens and receptors for complement components C3b and C3d in human-mouse hybrids.
Immunogenetics 3 465-471.

E.S.Curtoni,P.L.Mattiuz & R.M.Tosi (eds) 1967.
Histocompatibility Testing 1967.
Munksgaard,Copenhagen.

E.S.Curtoni,G.Scudeller,P.L.Mattiuz,M.Savi & R.Ceppelini 1972.
Anti-HLA antibody evolution in respect of planned transfusions.
Tissue Antigens 2 415-426.

S.Cwik,H.U.Cichon & D.O.Schmid 1979.
Serologically defined B-lymphocyte antigens in cattle
Proc.16th Int.Conf.Anim.Blood Grps.Biochem.Polymorphism (in press). Cited by Spooner et al. 1979 op.cit.

R.H.van Dam,C.B.van Werkhoven,J.A.van der Donk & J. Goudswaard 1976.
Histocompatibility in ruminants.1.The production and evaluation of alloantibodies for GL-A typing in goats.
J.Immunogenetics 3 237-244.

R.H.van Dam,J.D'Amaro,P.J.S.van Kooten,J.A.van der Donk & J.Goudswaard 1979.
The histocompatibility complex GLA in the goat.

Anim.Blood Grps.Biochem.Genet. 10 121-124.

J.Dausset 1958.
Iso-leuco-anticorps.
Acta Haematol. 20 156-166.

J.Dausset 1970.
The HLA system.Genetic and biological implications.
Acta Path.Microbiol.Scand. 78 529-546.

J.Dausset & J.Colombani (eds) 1973.
Histocompatibility Testing 1972.
Munksgaard,Copenhagen.

J.Dausset,J.Colombani & M.Colombani 1961.
Study of leukopenias and thrombocytopenias by the direct antiglobulin consumption test on leukocytes and/or platelets.
Blood 18 672-690.

J.Dausset,P.Ivanyi,J.Colombani,N.Feingold & L.Legrand 1967.
The Hu-1 system.
Histocompatibility Testing 1967 E.S.Curtoni,P.L. Mattiuz & R.M.Tosi (eds) Munksgaard,Copenhagen.pp189-202.

J.Dausset,P.Ivanyi & D.Ivanyi 1965.
Tissue alloantigens in humans:Identification of a complex system (Hu-1).
Histocompatibility Testing 1965 H.Balner,F.J.Cleton & J.G.Eernisse (eds) Munksgaard,Copenhagen pp51-62.

J.Dausset & A.Nenna 1952.
Présence d'une leucoagglutinine dans le sérum d'un cas d'agranulocytose chronique.
C.R.Soc.Biol. 146 1539-1541.

J.Dausset & A.Svejgaard (eds) 1975.
HLA and Disease.
Inserm,Paris.

C.S.David 1977.
The major histocompatibility system of the mouse.
The Major Histocompatibility System in Man and Animals. D.Gotze (ed) Springer-Verlag Berlin Heidelberg.pp255-290.

C.S.David 1979.
The genetic fine structure of H-2D end (abstr).
Immunogenetics 9 307-308.

C.S.David,D.C.Shreffler & J.A.Frelinger 1973a.
New lymphocyte antigen system (Lna) controlled by the Ir region of the mouse H-2 complex.
Proc.Natl.Acad.Sci. 70 2509-2514.

C.S.David,D.C.Shreffler,D.B.Murphy & J.Klein 1973b.
Serological cross-reaction between H-2D and H-2K region antigens.
Transplant.Proc. 5 287-293.

D.L.Davies & B.J.Alkins 1974.
What abrogates heart transplant rejection in immunological enhancement?
Nature 247 294-297.

J.R.Dawson,S.S.Shasby & D.B.Amos 1974.
The serological detection of HL-A antigens in human milk.

Tissue Antigens 4 76-82.

N.K.Day,L.Degos,E.Beth,M.Sassportes,R.Gharbi & G.Giraldi 1976.
C8 deficiency in a family with xeroderma pigmentosum Lack of linkage to the HL-A region.
Cited by B.Dupont et al. 1977 op.cit.

N.K.Day,P.Rubinstein,M.De Bracco,B.Moncada,J.A.Hansen, B.Dupont,M.Thomsen,A.Svejgaard & C.Jersild 1975.
Hereditary Clr deficiency:Lack of linkage to the Hl-A region in two families.
Histocompatibility Testing 1975 F.Kissmeyer-Nielsen (ed) Munksgaard,Copenhagen pp960-962.

J.M.Delâge,P.Bergeron,J.Simard,G.Lehner-Netsch & E.Prochazka 1977.
Hereditary C7 deficiency.Diagnosis and HLA studies in a French-Canadian family.
J.Clin.Invest. 60 1061-1069.

T.L.Delovitch & H.O.McDevitt 1975.
Isolation and characterisation of murine Ia antigens.
Immunogenetics 2 39-52.

P.Démant & C.Neauport-Sautes 1978.
The H-2L locus and the system of H-2 specificities.
Immunogenetics 7 295-311.

M.Dewar 1965.
An Introduction to Modern Chemistry.
Athlone Press,London.

C.Dewitt & M.McCullough 1977.
Lymphocyte membrane antigens in the rat:Two serologically determined loci at the MHC and three new minor histocompatibility loci.
Transplant. Proc 9 625-627.

E.Dickmeiss,B.Soeberg & A.Svejgaard 1977.
Human cell-mediated cytotoxicity against target cells is restricted by HLA.
Nature 270 526-528.

M.E.Dorf,H.Balner & B.Benacerraf 1975.
Mapping of the immune response genes in the major histocompatibility complex of the rhesus monkey.
J.Exp.Med 142 673-693.

M.E.Dorf,H.Balner,M.L.De Groot & B.Benacerraf 1974.
Histocompatibility-linked immune response genes in the rhesus monkey.
Transplant. Proc. 6 119-123.

M.E.Dorf,E.K.Toth & H.Balner 1972.
Cross-reactions of HL-A antibodies.V.Relationships between the human HL-A11 and chimpanzee ChW-11 specificities.
Tissue Antigens 2 461-472.

R.W.Doughty & K.Gelsthorpe 1974.
An initial investigation of lymphocyte antibody activity through pregnancy and in eluates prepared from placental material.
Tissue Antigens 4 291-298.

R.W.Doughty & K.Gelsthorpe 1976.
Some parameters of lymphocyte antibody activity through

pregnancy and further eluates of placental material.
Tissue Antigens 8 43-48.

W.R.Duncan & J.W.Streilein 1976.
Definition and analysis of the major histocompatibility complex in Syrian hamsters.
Ph.D Dissertation,Southwestern Medical School,Dallas Texas. Cited by Duncan et al. 1977 op.cit.

W.R.Duncan & J.W.Streilein 1978a.
Analysis of the major histocompatiblity complex in Syrian hamsters.1.Skin graft rejection,graft-versus-host reactions,mixed lymphocyte reactions and immune response genes in inbred strains.
Transplantation 25 12-16.

W.R.Duncan & J.W.Streilein 1978b.
Analysis of the major histocompatiblity complex in Syrian hamsters II.Linkage studies.
Transplantation 25 17-22.

W.R.Duncan,J.W.Streilein & P.Iványi 1977.
The major histocompatibility system of Syrian hamsters.
The Major Histocompatibility System in Man and Animals. D.Götze (ed) Springer-Verlag Berlin Heidelberg.pp183-189.

B.Dupont,R.A.Good,G.Hauptmann,I.Schreuder & M.Seligmann 1977.
Immunopathology,immunodeficiencies and complement deficiencies.
HLA and Disease.J.Dausset & A.Svejgaard (eds). Munksgaard,Copenhagen.

R.W.Dutton 1966.
Spleen cell proliferation in response to homologous antigens studied in congenic resistant strains of mice.
J.Exp.Med.123 665-671.

A.Ebringer 1978.
The link between genes and disease.
New Scientist 79 865-867.

A.Ebringer 1979.
Anklosing spondylitis,immune response genes and molecular mimicry.
Lancet 1 1186.

M.Edidin 1972.
The tissue distribution and cellular location of transplantation antigens.
Transplantation Antigens B.D.Kahan & R.A.Reisfield (eds) Academic Press,New York pp125-140.

N.Ehlers & S.Ahrons 1971.
The effect of histocompatibility upon the corneal immune reaction after intralamellar allotransplantation in rabbits.
Tissue Antigens 1 23-31.

W.L.Elkins & J.Palm 1966.
Identification of a single strong histocompatibility locus in the rat by normal spleen-cell transfer.
Ann.N.Y.Acad.Sci. 129 573-580.

L.Ellman,I.Green,W.J.Martin & B.Benacerraf 1970.

Linkage between the poly-L-lysine gene and the locus controlling the major histocompatibility antigens in strain 2 guinea pigs.
Proc.Natl.Acad.Sci. 66 322-328.

M.Elves 1974.
A study of the transplantation antigens on chrondrocytes from articular cartilage.
J.Bone Jt.Surg. 56B 178-185.

M.W.Elves 1976.
Flourochromatic cytotoxic test.
NIAID Manual of Tissue Typing Techniques.
J.G.Ray,D.B.Hare,P.D.Pedersen & D.I.Mullally (eds).
DHEW Publication No. (NIH) 78-545 U.S.A.pp37-39.

C.P.Engelfreit & E.van den Berg-Loonen 1976.
Development of monospecific antisera.
NIAID Manual of Tissue Typing Techniques.
J.G.Ray,D.B.Hare,P.D.Pedersen & D.I.Mullally (eds)
DHEW Publication No. (NIH) 78-545 U.S.A.pp40-41.

C.P.Engelfreit & A.Britten 1966.
Cytotoxic antibodies against leucocytes.
Vox Sang. 11 334-344.

R.B.Epstein & B.T.Bennet 1973.
Immunologic aspects of transmissable venereal tumour of canines (abstr).
Tissue Antigens 3 169.

A.van Es & H.Balner 1978.
RhLA complex of rhesus monkeys.X.Implications of the association between D Ial locus antigens.
Tissue Antigens 12 279-296.

D.L.Ewart & M.D.Cooper 1978.
Ia-like alloantigens in the chicken:Serologic characterisation and ontogeny of cellular expression.
Immunogenetics 7 521-535.

J.Farrant,S.C.Knight,L.E.McGann & J.O'Brien 1974.
Optimal recovery of lymphocytes and tissue culture cells following rapid cooling.
Nature 249 452-453.

W.P.Faulk & A.Temple 1976.
Distribution of B-2microglobulin and HLA in chorionic villi of human placentae.
Nature 262 799-802.

M.Fellous & J.Dausset 1970.
Probable haploid expression of HL-A antigens on human spermatozoa.
Nature 225 191-193.

G.Ferrara,R.Tosi,A.Longo,A.Castellani,C.Viviani & G.Carminati 1978a
'Silent' alleles at the HLA-C locus.
J.Immunol. 121 731-735.

G.B.Ferrara,R.Tosi,G.Azzolina,G.Carminati & A.Longo 1972.
Production of isoantisera for HL-A typing by planned immunisation.A four year's experiment.
Symp.Ser.Immunobiol.Std. 18 20-26.

G.B.Ferrara,R.M.Tosi,A.Longo,G.Azzolina & G.Carminati 1975.

Low immunogenicity of the third HL-A series.
Transplantation 20 340-343.

G.B.Ferrara,R.Tosi,A.Longo,A.Castellani,C.Viviani & G.Carminati 1978b.
A safe blood transfusion procedure for immunisation against major histocompatibility complex determinants in man.
Transplantation 26 150-152.

A.Ferreira & V.Nussenweig 1975.
Genetic linkage between serum levels of the third component of complement and the H-2 complex.
J.Exp.Med. 141 513-517.

A.Ferreira & V.Nussenweig 1976.
Control of C3 levels in mice during ontogeny by a gene in the central region of the H-2 complex.
Nature 260 613-615.

S.Ferrone 1977.
Rabbit complement in the lymphocytotoxicity test.
Tissue Antigens 9 223-226.

S.Ferrone,N.R.Cooper,M.A.Pellegrino & R.A. Reisfield 1971.
The lymphocytotoxic reaction:The mechanism of rabbit complement action.
J.Immunol. 107 939-947.

S.Ferrone,M.R.Mickey,P.I.Terasaki,R.A.Reisfield & M.A. Pellegrino 1976.
Humoral sensitization in parous women:Cytotoxic antibodies to non-HL-A antigens.
Transplantation 22 61-68.

S.Ferrone & M.A.Pellegrino 1973.
HL-A antigens,antibody and complement in the lymphocytotoxic reaction.
Contemp.Topics Molec.Immunol. 2 185-235.

S.Ferrone,R.M.Tosi & D.Centis 1967.
Anticomplementary factors affecting the lymphocytotoxicity test.
Histocompatibility Testing 1967 E.S.Curtoni,P.L.Mattiuz & R.M.Tosi (eds) Munksgaard,Copenhagen pp357-364.

H.Festenstein 1978.
Unpublished observations.
Cited by Morris et al. 1978 op.cit.

H.Festenstein,K.Abbasi,J.A.Sachs & R.T.D.Oliver 1972.
Serologically undetectable immune responses in transplantation.
Transplant.Proc. 4 219-222.

H.Festenstein & K.Halim 1976.
HLA-D-locus determinants detected by sperm-lymphocyte culture.
Transplant.Proc. 9 1239-1241.

F.D.Finkelman,E.M.Shevach,E.S.Vitteta,I.Green & W.E.Paul 1975.
Guinea pig immune response-related histocompatibility antigens.Partial characterisation and distribution.
J.Exp.Med. 141 27-41.

R.A.Fisher 1946.

Statistical Methods for Research Workers.
Oliver and Boyd,Edinburgh. 10th Ed.

R.A.Fisher 1930.
The Genetical Theory of Natural Selection.
Dover Publications,New York. 2nd.Ed. 1958.

L.Flaherty 1978.
Genes of the Tla region:The new Qa system of antigens.
Origins of Wild Mice. H.C.Morse (ed) Academic Press, New York.pp409-422.

R.Folger & H.Hines 1976.
Bovine lymphocyte antigens:Serological relationships with erythrocyte and spermatozoan antigens.
Anim.Blood Grps.Biochem.Genet. 7 137-145.

C.H.J.Ford 1973.
Ph.D Thesis.
University of London.

C.H.J.Ford 1974.
A serological investigation of sheep leucocyte antigens.
J.Immunogenetics 1 345-354.

C.H.J.Ford 1975.
Genetic studies of sheep leucocyte antigens.
J.Immunogenetics 2 31-40.

C.H.J.Ford & M.W.Elves 1974.
The production of cytotoxic antileucocyte antibodies by parous sheep.
J.Immunogenetics 1 259-264.

J.A.Frelinger,J.E.Niederhuber,C.S.David & D.C.Shreffler 1974.
Evidence for the expression of Ia (H-2I associated) antigens on thymus derived lymphocytes.
J.Exp.Med. 140 1273-1284.

S.M.Fu,N.Chiorazzi,C.Y.Wang,G.Montzeri,H.G.Kunkel,H.S.Ko & A.B.Gottleib 1978.
Ia-bearing T lymphocytes in man.Their identification and role in the generation of allogeneic helper activity.
J.Exp.Med. 148 1423-1428.

S.Fu,H.G.Kunkel,H.P.Brusman,F.H.Allen & M.Fotino 1974.
Evidence of linkage between HL-A histocompatibility genes and those involved in the synthesis of the second component of complement.
J.Exp.Med. 140 1108-1111.

S.M.Fu,R.Stern,H.G.KunKel,B.Dupont,J.A.Hansen,N.K.Day, R.A.Good,C.Jersild & M.Fotino 1975.
Mixed lymphocyte culture determinants and C2 deficiency:LD-7a associated with C2 deficiency in four families.
J.Exp.Med.142 495-506.

T.C.Fuller 1976.
A simple inexpensive procedur for freezing,storage and recovery of viable human lymphocytes.
NIAID Manual of Tissue Typing Techniques.
J.G.Ray,D.B.Hare,P.D.Pedersen & D.I.Mullally (eds)

DHEW Publication No. (NIH) 78-545 U.S.A.pp190-195.

D.L.Gasser 1977.
Current status of rat immunogenetics.
Adv.Immunol. 25 93-139.

D.L.Gasser,C.M.Newlin,J.Palm & N.K.Gonatas 1973.
Genetic control of susceptibility to experimental allergic encephalitis in rats.
Science 181 872-873.

A.F.Geczy & A.L.de Weck 1976.
The major histocompatibility complex of the guinea pig.III.Relationship between mixed leucocyte reactivity and serologicallly-defined phenotypes of the GPLA B locus and I region.
J.Immunogenetics 3 191-198.

A.F.Geczy & A.L.de Weck 1977.
The major histocompatibility system of the guinea pig.
The Major Histocompatibility System in Man and Animals.D.Götze (ed) Springer-Verlag Berlin Heidelberg pp193-206.

T.Gedde-Dahl,P.Teisberg & E.Thorsby 1974.
C3 polymorphism:Genetic linkage relations.
Clin.Genet. 6 66-72.

M.C.Gelfand,D.H.Sachs,R.Liebermann & W.E.Paul 1974.
Ontogeny of B lymphocytes.III.H-2 linkage of a gene controlling the rate of appearance of complement receptor lymphocytes.
J.Exp.Med. 139 1142-1153.

C.M.Giles,T.Gedde-Dahl,E.B.Robson,E.Thorsby B.Olaisen A.Arnason,F.Kissmeyer-Nielsen & I.Schreuder 1976.
Rg^a(rodgers) and the HLA region:Linkage and associations.
Tissue Antigens 8 143-149.

T.J.Gill & H.W.Kunz 1976.
Genetic studies in inbred rats.VII.Tentative model for the major histocompatibility complex.
J.Immunogenetics 3 139-143.

M.B.Goldman & J.N.Goldman 1975.
Relationships of levels of early components of complement to the H-2 complex in mice (abstr).
Fed.Proc. 34 979.

E.S.Golub 1978.
The Cellular Basis of the Immune Response.
Sinauer,Sunderland Mass. U.S.A.

P.N.Goodfellow,C.J.Barnstable,W.F.Bodmer,D.Snary & M.J.Crumpton 1976.
Expression of HLA system antigens on placenta.
Transplantation 22 595-603.

P.N.Goodfellow,E.A.Jones,V.van Heyningen,E.Solomon,M.Bobrow V.Miggiano & W.F.Bodmer 1975.
The B-2microglobulin gene is on chromosome 15 and not in the HL-A region.
Nature 254 267-268.

P.N.Goodfellow,E.Jones,V.van Heyningen,E.Solomon,R.Kennett M.Bobrow & W.F.Bodmer 1974.

Linkage relationships of the HL-A system and B-2microglobulin.
Cytogenet.Cell.Genet. 14 332-337.

P.N.Goodfellow & R.O.Payne 1978.
Serological studies of Cw1 and Cw3 expressed on the same haplotype.
Tissue Antigens 11 350-356.

R.C.Goodlin & L.A.Herzenberg 1964.
Pregnancy induced haemagglutinins to paternal H-2 antigens in multiparous mice.
Transplantation 2 357-361.

P.A.Gorer & P.O'Gorman 1956.
The cytotoxic activity of isoantibodies in mice.
Transplant.Bull. 3 142-143.

D.Götze (ed) 1977.
The Major Histocompatibility System in Man and Animals. Springer-Verlag,Berlin Heidelberg.

D.Götze 1975.
T(Ia+) and B(Iab)-cell alloantigens determined by the H-2 linked I region in mice.
Immunogenetics 1 495-507.

E.Goulmy,A.Termijtelen,B.A.Bradley & J.J.van Rood 1976.
HLA restriction of non HLA-A,B,C and D cell mediated lympholysis (CML).
Tissue Antigens 8 317-326.

E.Goulmy,A.Termijtelen,B.A.Bradley & J.J.van Rood 1977.
Y-antigen killing by T cells of women is restricted by HLA.
Nature 266 544-545.

J.L.Gowans & E.J.Knight 1964.
The route of recirculation of lymphocytes in the rat.
Proc.Roy.Soc.B 159 257-282.

H.Grosse-Wilde,P.Baumann,B.Netzel,H.J.Kolb,R.Wank,W.Mempel & E.D.Albert 1973.
One way non-stimulation in MLC related to DL-A homozygosity.
Transplant.Proc. 5 1567-1571.

H.Grosse-Wilde,H.M.Vriesendorp,B.Netzel,W.Mempel,H.J.Kolb R.Wank & E.D.Albert 1975.
Immunogenetics of 7 LD alleles of the DLA complex in mongrels,beagles and labradors.
Transplant.Proc. 7 Suppl.1 159-169.

E.Günther,J.Balcarova,K.Hala,E.Rude & T.Hruba 1974.
Evidence for an association between immune responsiveness of chicken to (T,G)-A--L and the major histocompatibility system.
Eur.J.Immunol. 4 548-553.

E.Günther & O.Stark 1977.
The major histocompatibility system of the rat (Ag-B or H-1 system).
The Major Histocompatibility System in Man and Animals D.Gotze (ed) Springer-Verlag,Berlin Heidelberg. pp207-253.

E.Günther & O.Stark 1978.

At least two loci of the major histocompatibility complex determine mixed lymphocyte stimulation in the rat.
Tissue Antigens 11 465-470.

E.Günther,O.Stark & C.Koch 1978.
Genetic definition of I region-determined antigens of the rat major histocompatibility complex.
Eur.J.Immunol. 8 206-212.

K.Hála 1977.
The major histocompatibility system of the chicken.
The Major Histocompatibility System in Man and Animals
D.Götze (ed) Springer-Verlag,Berlin Heidelberg
pp291-312.

K.Hála,M.Vilhelmová & J.Hartmanová 1975.
Probable crossing-over between 2 alleles of the B system in chickens.
Folia Biol. 21 363-365.

K.Hála,M.Vilhelmová & J.Hartmanová 1976.
Probable crossing over in the B blood group system of chickens.
Immunogenetics 3 97-103.

K.Halim & H.Festenstein 1975.
HLA-D on sperms.
Lancet 2 1255-1256.

C.Hammerberg & J.Klein 1975.
Linkage disequilibrium between H-2 and t complexes in chromosome 17 of the mouse.
Nature 258 296-299.

G.J.Hämmerling 1976.
Tissue distribution of Ia antigens and their expression on lymphocyte subpopulations.
Transplant.Rev. 30 64-82.

G.J.Hämmerling,B.D.Deak,G.Mauve,U.Hämmerling & H.O.McDevitt 1974.
B lymphocyte alloantigens controlled by the I region of the major histocompatibility complex in mice.
Immunogenetics 1 68-81.

G.J.Hämmerling,U.Hämmerling & L.Flaherty 1979.
Qat-4 and Qat-5,new murine T-cell antigens governed by the Tla region and identified by monoclonal antibodies.
J.Exp.Med. 150 108-116.

G.J.Hämmerling,G.Mauve,E.Goldberg & H.O.McDevitt 1975.
Tissue distribution of Ia antigens:Ia on spermatozoa, macrophages and epidermal cells.
Immunogenetics 1 428-438.

J.Hanks & J.H.Wallace 1958.
Determination of cell viability.
Proc.Soc.Exp.Biol. 98 188-192.

M.P.Hanson,J.N.van Zandt & G.R.J.Law 1967.
Differences in susceptibility to Marek's disease in chickens carrying two different B locus blood group alleles (abstr).
Poult.Sci. 46 1268.

R.E.Harris & R.E.Lordon 1976.
The association of maternal lymphocytotoxic antibodies with obstetric complications.
Obstet.Gynecol. 48 302-304.

R.Harris,J.Wentzel,H.Cocking,H.Dodsworth & E.O.Ukaejiofo 1970.
Errors in allograft donor typing:A modified microcytotoxic test.
Histocompatibility Testing 1970 P.I.Terasaki (ed) Munksgaard,Copenhagen.pp603-616.

R.Harris & J.Zervas 1969.
Reticulocyte HL-A antigens.
Nature 221 1062-1063.

V.Hauptfeld,D.Klein & J.Klein 1973.
Serological identification of an Ir-region product.
Science 181 167-169.

V.Hauptfeld & J.Klein 1975.
Molecular relationships between private and public antigens as determined by antigen redistribution method.
J.Exp.Med. 142 288-298.

M.Hauptfeld & J.Klein 1976.
The H-2 complexes of inbred and wild mice are organised in a similar fashion.
Immunogenetics 3 603-607.

G.Hauptmann,M.M.Tongio,H.Grosse-Wilde & S.Mayer 1977.
Linkage between C2 deficiency and the HLA-A10,B18, Dw2/BfS haplotype in a French family.
Immunogenetics 4 557-565.

S.J.Hausman & J.Palm 1973.
Variable expression of Ag-B and non-Ag-B histocompatibility antigens on cultured rat cells of different histological origin.
Transplantation 16 313-324.

L.A.Herzenberg,D.W.Bianchi,J.Schroder,H.M.Cann & G.M.Iverson 1979.
Fetal cells in the blood of pregnant women: Detection and enrichment by flourescence-activated cell sorting.
Proc.Natl.Acad.Sci. 76 1453-1455.

L.A.Herzenberg & B.Gonzales 1962.
Appearance of H-2 agglutinins in outcrossed female mice.
Proc.Natl.Acad.Sci. 48 570-573.

C.Hiller,M.Bischoff,A.Schmidt & K.Bender 1978.
Analysis of the HLA-ABC linkage disequilibrium: Decreasing strength of gametic association with map distance.
Hum.Genet. 41 301-312.

A.A.Hirata & P.I.Terasaki 1972.
Masking of human transplantation antigens by diverse substances.
J.Immunol. 108 1542-1550.

H.Hirschberg,S.A.Evensen,T.Henriksen & E.Thorsby 1974.
Stimulation of human lymphocytes by allogeneic endothelial cells in vitro.

Tissue Antigens 4 257-261.

H.Hirschberg,A.Kaakinen & E.Thorsby 1976.
Presence of HLA-D determinants on human macrophages.
Nature 263 63-64.

H.Hirschberg & E.Thorsby 1975.
Lymphocyte activating alloantigens on human epidermal cells.
Tissue Antigens 6 183-194.

W.H.Hitzig & R.Gitzelmann 1959.
Transplacental transfer of leukocyte agglutinins.
Vox Sang. 4 445-455.

I.Hložánek & K.Hála 1969.
The detection of a B red blood cell allele in chick embryo fibroblasts.
Folia Biol. 15 474-477.

C.K.Ho,L.A.Babiuk & B.T.Rouse 1978.
Immune effector cell activity in canines: Failure to demonstrate genetic restriction in direct anti-viral cytotoxicity.
Infect.Immun. 19 18-26.

M.Hobart 1977.
Complementary genetics.
Nature 266 681-682.

M.J.Hobart,P.J.L.Cook & P.J.Lachman 1977.
Linkage studies with C6.
J.Immunogentics 4 423-428.

M.J.Hobart,V.Joysey & P.Lachman 1978.
Inherited structural variation and linkage relationships of C7.
J.Immunogenetics 5 157-163.

M.J.Hobart & P.J.Lachmann 1976.
Allotypes of complement components in man.
Transplant.Rev. 32 26-42.

G.Hoecker,S.Counce & P.Smith 1954.
The antigens determined by the H-2 locus: A rhesus-like system in the mouse.
Proc.Natl.Acad.Sci. 40 1040-1051.

B.Hogan,M.Fellous,P.Avner & F.Jacob 1977.
Isolation of a human teratoma cell line which expresses F9 antigen.
Nature 270 515-518.

P.J.Hogarth 1978.
Biology of Reproduction.
Blackie,Glasgow London.

J.Hors,J.L.Preud'homme,M.Toulze-Zapateria,J.Guilet-Bigot, J.Roy & J.Dausset 1973.
A simplified method for freezing lymphocytes in nitrogen vapours.
Transplantation 15 417-419.

V.Hruban,M.Simon,J.Hradecky & F.Jilek 1976.
Linkage of the pig main histocomptibility complex and the J blood group system.
Tissue Antigens 7 267-271.

T.H.Iha,G.Gerbrandt,W.Bodmer,D.McGary & W.Stone 1973.

Cross reactions of cattle lymphocytic sera with HL-A and other human antigens.
Tissue Antigens 3 291-302.

P.Iványi 1977a.
The major histocompatibility system of the pig.
The Major Histocompatibility System in Man and Animals. D.Götze (ed) Springer-Verlag Berlin Heidelberg.pp168-189.

P.Iványi 1977b.
The major histocompatibility system of the rabbit.
The Major Histocompatibility System in Man and Animals. D.Götze (ed) Springer-Verlag Berlin Heidelberg.pp174-189.

P.Iványi,E.Gyódi,G.Györffy,H.Pavljuková,E.Ivasková & G. Petrányi 1977.
Cytotoxic effect of anti-H-2 and anti-Ia antisera on human B and T cells.
Scand.J.Immunol. 6 431-437.

P.Iványi,H.Pauljuková & E.Ivasková 1976.
H-2/HLA cross-reactions.Absorption analysis of cytotoxic anti-human activity in anti-H-2 mouse sera.
Transplantation 22 612-618.

E.Ivašková,J.Dausset & P.Iványi 1972.
Cytotoxic reactions of anti-H-2 sera with human lymphocytes.
Folia Biol. 18 194.

E.Ivašková,P.Iványi,H.Bavarová & I.Egorov 1976.
Cross-reactions of some anti-H-2 sera with HLA-A9.
Folia Biol. 22 58-59.

E.Ivašková,P.Iványi,M.Micková & P.Démant 1975.
Cross-reactions between the major histocompatibility system of man and mice (HLA and H-2).Cytotoxic action of anti-H-2 sera on lymphocytes.
Tissue Antigens 6 286-311.

H.E.Jasin 1977.
Absence of the eigth component of complement in association with systemic lupus erythematosus-like disease.
J.Clin.Invest. 60 709-715.

K.G.Jensen & F.Kissmeyer-Nielsen 1969.
Immunology of Leukocytes.
Progress in Clinical Pathology. M.Stefanini (ed) vol.2 Grune & Stratton Inc.,New York.

M.H.Johnson 1974.
Foeto-maternal interactions.
Progress in Immunology II 2 L.Brent & J.Holborrow (eds) North Holland,Amsterdam. pp400-403.

M.Johson,B.Hattler,K.Alexander & C.Carrier 1971.
Effect of granulocyte concentration of mixed lymphocyte culture (abstr).
Fed.Proc. 30 466.

Joint Report 1970.
Histocompatibility Testing 1970. P.I.Terasaki (ed) Munksgaard,Copenhagen. pp2-47.

Joint Report 1973.

First international workshop on canine Immunogenetics Tissue Antigens 3 145-163.

T.G.Jones,K.L.G.Goldsmith & I.M.Anderson 1961.
Maternal and neonatal platelet antibodies in a case of congenital thrombocytopenia.
Lancet 2 1008-1009.

A.Jongsma,H.van Someren,A.Westerveld,A.Hagemeijor & P.Pearson 1973.
Localisation of genes on human chromosomes using Human -Chinese hamster somatic cell hybrids.Assignment of PGM-3 to chromosome C6 and regional mapping of the PGD,PGM-1 and PEP-C genes to chromosome A1.
Humangenetik. 20 195-202.

V.C.Joysey 1969.
A modified microcytotoxic test for lymphocyte typing using siliconed glass slides and ordinary phase contrast microscopy.
Proc.Symp.Transplant.Antigens Tissue Typing. M.W.Elves & N.W.Nisbet (ed). Oswestry,England.

V.C.Joysey & E.C.Wolf 1978.
HLA-A,-B and -C antigens.Their serology and cross-reactions.
Brit.Med.Bull. 34 217-222.

R.Kachru & K.Mittal 1975.
Serological detection of HL-A antigens in human mammary secretions.
Histocompatibility Testing 1975 F.Kissmeyer-Nielsen (ed). Munksgaard,Copenhagen. pp404-413.

B.D.Kahan 1967.
Cutaneous hypersensitivity reactions of guinea pigs to proteinaceous transplantation antigen.
J.Immunol. 99 1121-1127.

B.D.Kahan & R.A.Reisfield 1967.
Eletrophoretic purification of a water-soluble guinea pig transplantation antigen.
Proc.Natl.Acad.Sci. 58 1430-1437.

N.Kaliss & M.K.Darg 1964.
Immune response engendered in mice by multiparity.
Transplantation 2 416-425.

F.S.Kantor,A.Ojeda & B.Benacerraf 1963.
Studies on artificial antigens.1.Antigenicity of DNP-polylysine and DNP-copolymers of lysine and glutamic acid in guinea pigs.
J.Exp.Med. 117 55-69.

M.Katagiri,T.Natori,N.Tanagaki,V.P.Kreiter & D.Pressman 1975a.
Papain-solubilized Ag-B antigens.1.Isolation and characterisation of two components composing Ag-B antigens.
Transplantation 19 230-239.

M.Katagiri,N.Tanigaki & D.Pressman 1975b
Papain-solubilised Ag-B antigens.II.Characterization of small sized Ag-B molecules.
Transplantation 20 135-141.

D.H.Katz,T.Hamaoka,M.E.Dorf & B.Benacerraf 1973.
Cell interactions between histoincompatible T

and B lymphocytes. The H-2 gene complex determines sucessful physiolgic lymphocyte interactions. Proc.Natl.Acad.Sci. 70 2624-2628.

P.M.Khan,W.R.T.Los,J.A.van der Does & R.A.Epstein 1976. Manuscript in preparation. Cited by Vriesendorp et al. 1977 op.cit.

E.S.Kimball,J.E.Coligan & T.J.Kindt 1979. Structural characterisation of antigens encoded by rabbit RLA-11 histocompatibility genes. Immunogenetics 8 201-211.

B.Kindred & D.C.Shreffler 1972. H-2 dependance of co-operation between T and B cells in vivo.

F.Kissmeyer-Nielsen 1975. Histocompatibility Testing 1975. Munksgaard,Copenhagen.

F.Kissmeyer-Nielsen & K.E.Kjerbye 1967. Lymphocytotoxic microtechnique.Purification of lymphocytes by flotation. Histocompatibility Testing 1967 E.S.Curtoni,P.L. Mattiuz & R.M.Tosi (eds). Munksgaard,Copenhagen. pp381-383.

F.Kissmeyer-Nielsen,A.Svejgaard,S.Ahrons & L.S.Nielsen 1969. Crossing-over within the HL-A system. Nature 224 75-76.

J.Klein 1970. Histocompatibility-2 (H-2) polymorphism in wild mice. Sciénce 168 1362-1364.

J.Klein 1971. Private and public antigens of the mouse H-2 system. Nature 229 635-637.

J.Klein 1972. Histocompatibility-2 system in wild mice.1. Identification of five new H-2 chromosomes. Transplantation 13 291-299.

J.Klein 1975. Biology of the Mouse Histocompatibility-2 Complex. Springer-Verlag,Berlin Heidelberg.

J.Klein 1977. Evolution and function of the major histocompatibility system:Facts and speculations. The Major Histocompatibility System in Man and Animals. D.Gotze (ed) Springer-Verlag Berlin Heidelberg.pp339-378.

J.Klein 1979a Dialogue between a teacher and his student concerning H-2 nomenclature. Immunogenetics 8 377-378.

J.Klein 1979b The major histocompatibility system of the mouse. Science 203 516-521.

J.Klein,F.H.Bach,H.Festenstein,H.O.McDevitt,D.C.Shreffler, G.D.Snell & J.H.Stimpfling 1974. Genetic nomenclature for the H-2 complex of the mouse.

Immunogenetics 1 184-188.

S.C.Knight & J.Farrant 1978.
Symposium on viability assays-Synopsis papers. The problem of assaying the recovery of lymphocytes and other blood cells after freezing and thawing.
Cryobiol. 15 230-231.

C.Koch & M.Simonsen 1977.
Immune response genes in chickens.Antibody response to TGAL and GT.
Immunogenetics 5 161-170.

G.Kohler & C.Milstein 1975.
Continous cultures of fused cells secreting antibody of predefined specificity.
Nature 256 495-497.

H.I.Kohn,J.Klein,R.W.Melvoid,S.G.Nathenson,D.Pious & D.C.Shreffler 1978.
The first H-2 mutant workshop.
Immunogenetics 7 279-294.

J.Kömpf,S.Bissbort & F.Schunter 1976.
Confirmation of linkage between the loci for HL-A and glyoxylase 1.
Hum.Genet. 32 197-198.

M.Kronke,U.Hadding,A.F.Geczy,A.De Weck & D.Bitter-Suermann 1977.
Linkage of guinea pig Bf and C4 to the GPLA
J.Immunol.119 2016-2018.

P.J.Lachmann,D.Grennan,A.Martin & P.Demant 1975.
Identification of Ss protein as murine C4.
Nature 258 242-243.

P.J.Lachmann & M.J.Hobart 1978.
Complement genetics in relation to HLA.
Brit.Med.Bull. 34 247-252.

P.Lalezari,M.Nussbaum,S.Gelman & T.H.Spaet 1959.
Neonatal neutropenia due to maternal isoimmunisation.
Clin.Res. 7 13-14.

P.Lalezari,M.Nussbaum,S.Gelman & T.H.Spaet 1960.
Neonatal neutropenia due to maternal isoimmunisation.
Blood 15 236-243.

L.U.Lamm,U.Friedrich,G.B.Petersen,J.Jorgensen,J.Nielsen A.J.Therkelsen & F.Kissmeyer-Nielsen 1974.
Assignment of the major histocompatiblity complex to chromosome NO.6 in a family with a pericentric inversion.
Hum.Hered. 24 273-284.

L.U.Lamm,A.Svejgaard & F.Kissmeyer-Nielsen 1971.
PGM-3:HLA is another linkage in man.
Nature New Biol. 231 109-111.

A.van Leeuwen,H.R.E.Schuit & J.J.van Rood 1973.
Typing for MLC (LD):11.The selection of nonstimulator cells by MLC inhibition tests using SD-identical stimulator cells (MISIS) and flourescence antibody studies.
Transplant.Proc. 5 1539-1542.

L.Leinwand,E.Nichols & F.H.Ruddle 1978.

Assignment of the gene glyoxylase 1 to chromosome 17 by somatic cell genetics. Biochem.Genet. 16 659-666.

S.Léon,H.M.Vriesendorp,A.Zweibaum,C.Fleche-Seban & G.Chevalier 1975a
Application à l'étude sérologique du système majeur d'histocompatibilité du chien (DLA) des propriétés antigéniques et immunogènes de la poudre acétonique du rate. C.R.Acad.Sci.D 281 1653-1656.

S.Léon,A.Zweibaum,H.M.Vreisendorp & B.J.Smid-Mercx 1975b
Production of tissue typing reagents in rabbits immunized with dog-spleen dry acetone powder. Transplant.Proc. 7 379-382.

V.Lepage,L.Degos & J.Dausset 1976.
A 'natural' anti-HLA-A2 antibody reacting with homozygous cells. Tissue Antigens 8 139-142.

P.Levine,L.Burnham,E.M.Katzin & P.Vogel 1941a
The role of iso-immunisation in the pathogenesis of erythroblastosis fetalis. Am.J.Obstet.Gynec. 42 925-937.

P.Levine,E.M.Katzin & L.Burnham 1941b.
Isoimmunisation in pregnancy.Its possible bearing on the etiology of erythroblastosis fetalis. J.Amer.Med.Ass. 116 825-827.

B.B.Levin,R.H.Stember & M.Fotino 1972.
Ragweed hayfever:genetic control and linkage to HL-A haplotypes. Science 178 1201-1203.

W.R.Levis & A.E.Miller 1972.
Leukocyte/skin cultures as a measure of histocompatibility in man. Lancet 2 357-360.

R.Lewontin 1974.
The Genetic Basis of Evolutionary Change. Columbia University Press,New York

J.Lightbody,D.Bernoco,V.C.Miggiano & R.Ceppelini 1971.
Cell mediated lympholysis in man after sensitisation of effector lymphocytes through mixed leucocyte cultures. G.Bact.Virol.Immunol. 64 243-254.

F.Lilly 1966.
The inheritance of susceptibility to the Gross leukemia virus in mice. Genetics 53 529-539.

K.F.Lindahl & B.Hausmann 1979.
Qed-1:A target for unrestricted T cell killing encoded distal to Qa-2 (abstr). Immunogenetics 9 323-324.

J.J.van Loghem,M.van der Hart,W.Hijmans & H.R.E.Schuit 1958.
The incidence and significance of complete and incomplete white cell antibodies with special reference to the use of the Coombs

consumption test.
Vox Sang. 3 203-223.

H.P.Lohrman,1.Novikons & R.G.Graw 1974.
Stimulatory capacity of human B and T lymphocytes in mixed lymphocyte culture.
Nature 250 144-146.

B.Low,L.Messster,S.Mansson & T.Lindholm 1974.
Crossing-over between the SD-2 (Four) and SD-3 (AJ) loci of the human major histocompatibility chromosomal region (abstr).
Tissue Antigens 4 405.

J.K.Lunney & D.H.Sachs 1978.
Transplantation in miniature swine.IV. Chemical characterisation of MSLA and Ia-like antigens.
J.Immunol. 120 607-612.

J.K.Lunney & D.H.Sachs 1979.
Transplantatio in miniature swine.V. Characterisation of Ia antigens.
J.Immunol. 122 623-627.

B.Luyet & J.Keane 1955.
A critical temperature range apparently characterized by sensitivity of bull semen to high freezing velocity.
Biodynamica 7 281-292.

A.Marshak,P.C.Doughty & D.B.Wilson 1977.
The control of specificity of cytotoxic T lymphocytes by the major histocompatibility complex (Ag-B) in rats and identification of a new alloantigen system showing no Ag-B restriction.
J.Exp.Med. 146 1773-1790.

W.H.Marshall,S.J.Rigo & S.Melman 1966.
Lymphocyte transformation and mitosis in vitro initiated by homologous macrophages.
Lancet 1 730-732.

J.Martinis & F.Bach 1977.
Human LD antigens are present on xenogeneic cells.
Nature 266 540-542.

D.W.Mason & G.G.Gallico 1978.
Tissue antigens and quantitation of Ia-like antigens in the rat.
Eur.J.Immunol. 8 741-748.

H.Matej 1970a
Further studies on antigenic differentiation of rabbit lymphocytes.
Arch.Immunol.Ther.Exp. 18 173-184.

H.Matej 1970b
Studies on histocompatibility in skin allotransplantation in rabbits.
Arch.Immunol.Ther.Exp. 18 315-326.

W.Mayr 1978.
Leucocyte antigens.
The Biochemical Genetics of Man. J.Brock & O.Mayo (eds) 2nd.Ed. Academic Press,London. pp373-420.

W.Mayr,D.Bernoco,M.De Marchi & R.Ceppelini 1973.
Genetic analysis and biochemical properties of

products of the third SD (AJ) locus of the HL-A region.
Transplant.Proc. 5 1581-1593.

W.R.Mayr,D.Mayr,J.Kompf,S.Bissbort & H.Ritter 1976.
Possible linkage of Hl-A and GLO.
Humangenetik 31 241-242.

P.Mazur 1976.
Mechanisms of injury and protection in cells and tissues at low temperatures.
Les Colloques de l'institut National de la Sante et de la Recherche Medicale. Cryoimmunology.
Inserm. 17-19 June 1976 62 37-60.

McBride 1979.
Unpublished observations.
Cited by Zinkernagel and Doherty 1979 op.cit.

J.N.McCormick,W.P.Faulk,H.Fox & H.H.Fudenberg 1971.
Immunohistological and elution studies of the human placenta.
J.Exp.Med. 133 1-18.

E.M.McDermid 1964.
Immunogenetics of the chicken.
Vox Sang. 9 249-267.

E.M.McDermid,N.S.Agar & C.K.Chai 1975.
Electrophoretic variation of red cell enzyme systems in farm animals.
Anim.Blood Grps.Biochem.Genet. 6 127-174.

A.J.McMichael,A.Ting,H.J.Zweerink & B.A.Askonas 1977.
HLA restriction of cell-mdiated lysis of influenza virus-infected human cells.
Nature 270 524-526.

C.J.M.Melief,M.van der Hart,C.P.Engelfreit & J.J.van Loghem 1967.
Immune adherence of leukocytes and fibroblasts derived from skin sensitized by cytotoxic leukocyte isoantibodies and complement to the surface of indicator cells.
Vox Sang. 12 374-389.

T.Meo,C.S.David,A.M.Rijnbeek,M.Nabholz,V.C.Miggiano & D.C.Shreffler 1975a.
Inhibition of mouse MLR by anti-Ia sera.
Transplant.Proc. 7 127-129.

T.Meo,T.Douglas & A.M.Rijnbeek 1977.
Glyoxylase 1 polymorphism in the mouse: A new genetic marker linked to H-2.
Science 198 311-313.

T.Meo,T.Krasteff & D.C.Shreffler 1975b.
Immunochemical characterisation of murine H-2 controlled Ss (serum substance) protein through identification of its human homologue as the fourth component of complement.
Proc.Natl.Acad.Sci. 72 4536-4540.

A.D.Merrit,B.H.Petersen,A.A.Biegel,D.A.Meyers,G.F.Brooks & M.E.Hodes 1976.
Chromosome 6:Linkage of the eigth component of complement (C8) to the histocompatibility region (HLA).
Cytogenet. Cell. Genet. 16 331-334.

R.S.Metzgar 1965.
The use of inhibition of mixed agglutination as a method for human tissue typing.
Histocompatibility Testing 1965. H.Balner,F.J.Cleton & J.G.Eernisse (eds). Munksgaard,Copenhagen. pp185-191.

R.S.Metzgar,J.Bertoglio,J.K.Anderson,G.D.Bonnard & F.W.Ruscetti 1979.
Detection of HLA-DRw (Ia-like) antigens on human T lymphocytes grown in tissue culture.
J.Immunol. 122 949-953.

M.P.Mi & N.E.Morton 1966.
Blood factor association.
Vox Sang. 11 434-449.

J.Michaelson 1978.
Cited by Flaherty 1978 op.cit.

J.Michaelson,L.Flaherty,E.Vitteta & M.D.Poulik 1977.
Molecular similarities between the Qa-2 alloantigen and other gene products of the 17th chromosome of the mouse.
J.Exp.Med. 145 1066-1070.

J.Middleton,M.C.Crookston,J.A.Falk,E.M.Robson,P.J.L.Cook J.R.Batchelor,J.Bodmer,G.B.Ferrara,H.Festenstein,R.Harris F.Kissmeyer-Nielsen,S.D.Lawler,J.A.Sachs & E.Wolf 1974.
Linkage of Chido and HL-A.
Tissue Antigens 4 366-373.

V.C.Miggiano,I.Bergen & J.R.L.Pink 1974.
The mixed leucocyte reaction in chickens:Evidence for control by the major histocompatibility complex.
Eur.J.Immunol. 4 397-401.

V.C.Miggiano,M.Nabholz & W.F.Bodmer 1970.
Detection of HL-A and other antigens on fibroblast micro-monolayers using a flourochromatic assay.
Histocompatibility Testing 1970 P.I.Terasaki (ed) Munksgaard,Copenhagen. pp623-630.

F.Milgrom,M.Palester,G.Woznicko & Z.Dudiack 1957.
Complement fixing leukocyte antibodies.
Vox Sang. 2 263-269.

P.Millot 1971.
Détermination d'antigènes lymphocytaires chez les ovins.
C.R.Acad.Sci.D 273 2028-2030.

P.Millot 1978.
The major histocompatibility complex of sheep (OLA) and two minor loci.
Anim.Blood Grps.Biochem.Genet. 9 115-121.

K.K.Mittal,M.R.Mickey,D.P.Singal & P.I.Terasaki 1968.
Serotyping for homotransplantation.XVIII.Refinement of microdroplet lymphocyte cytotoxicity test.
Transplantation 6 913-927.

K.K.Mittal,M.R.Mickey & P.I.Terasaki 1972.
Cross-reactive antibodies in 'duospecific' anti-HL-A antisera.
Symp.Ser.Immunobiol.Std. 18 165-170.

K.K.Mittal & P.I.Terasaki 1972.
Cross-reactivity in the HL-A system.

Tissue Antigens 2 94-104.

K.K.Mittal,K.P.Wolski,D.Lim.A.Gewurz,H.Gewurz & F.R.Schmid 1976.
Genetic independence between the HL-A system and deficits in the first and sixth components of complement.
Tissue Antigens 7 97-104.

J.R.Moraes & P.Stasny 1975.
Allo-antibodies to endothelial cell antigens.
Histocompatibility Testing 1975 F.Kissmeyer-Nielsen (ed). Munksgaard,Copenhagen. pp391-397.

P.J.Morris,J.R.Batchelor & H.Festenstein 1978.
Matching for HLA in transplantation.
Brit.Med.Bull. 34 259-262.

C.Mueller-Eckhardt,D.Heinrich & V.Rothenberg 1972.
Frequency and complexity of cross-reactive HL-A antibodies. Elution studies with platelets.
Symp.Ser.Immunobiol.Std. 18 171-178.

D.Murphy,L.A.Herzenberg,K.Okumura,L.A.Herzenberg & H.McDevitt 1976.
A new I region (I-J) marked by a locus (Ia-4) controlling surface determinants on suppressor T lymphocytes.
J.Exp.Med. 144 699-712.

D.B.Murphy & D.C.Shreffler 1975a
Cross-reactivity between H-2K and H-2D products.1.
Evidence for extensive and reciprocal serologic cross reactivity.
J.Exp.Med. 141 374-391.

D.B.Murphy & D.C.Shreffler 1975b
Cross reactivity between H-2K and H-2D products.III.
Effect of H-2K-H-2D cross-sensitization on skin graft survival.
Transplantation 20 38-48.

S.Naito,M.R.Mickey,A.J.Ebbin.J.Strauss & P.I.Terasaki 1970.
Fetal-maternal incompatibility.II.Direct cross-match studies on 38 children with birth defects.
Histocompatibility Testing 1970 P.I.Terasaki (ed) Munksgaard,Copenhagen. pp489-494.

S.G.Nathenson & S.E.Cullen 1974.
Biochemical properties and immunochemical-genetic relationships of mouse H-2 alloantigens.
Biochem.Biophys.Acta. 344 1-25.

S.G.Nathenson & T.Muramatsu 1971.
Properties of the carbohydrate portion of mouse alloantigen glycoproteins.
Glycoproteins of Blood Cells and Plasma. G.A.Jamieson & T.J.Greenwalt (eds). lippincott,Philadelphia,U.S.A. pp245-262.

T.Natori,T.Ohhashi,T.Kotani,M.Katagiri & M.Aizawa 1979.
The molecular identification of two serologically defined gene products of the rat major histocompatibility complex.
J.Immunol. 122 1911-1915.

D.Nelken,I.Cotten & I.Furcraig 1970.
A method to increase the sensitivity of the lymphocyte microcytotoxicity test.
Transplantation 10 346-347.

M.J.Newman & H.C.Hines 1979.
The production of foetally stimulated lymphocytotoxic antibodies by primiparous cows.
Anim.Blood Grps.Biochem.Genet. 10 87-92.

J.Oh & L.McLean 1975.
Comparative immunogenicity of HL-A antigens:A study in primiparas.
Tissue Antigens 5 33-37.

J.H.Oh,M.H.Gault,S.J.Helle & J.B.Dosseter 1972.
Developement of lymphocytotoxic antibodies in haemodialysis patients.
Vox Sang. 22 208-212.

S.Ohno 1970.
Evolution by Gene Duplication.
George Allen & Unwin,London.

R.T.D.Oliver,J.A.Sachs,H.Festenstein,G.D.Pegrum & J.F.Moorhead 1972.
Influence of HL-A matching,antigenic strength and immune responsiveness on outcome of 349 cadaver renal grafts.3 years experience of the London transplant group.
Lancet 2 1381-1385.

J.H.Olving,B.Olaisen,P.Teisberg,T.Gedde-Dahl & E.Thorsby 1977.
Non linkage between C6 and chromosome 6 markers.
Hum.Genet. 37 125-129.

G.O'Neil,S.Y.Yang,J.Tegoli,R.Berger & B.Dupont 1978.
Chido and Rodgers blood groups are distinct antigenic components of human complement C4.
Nature 273 668-670.

S.Ostrand-Rosenberg & C.Stormont 1974.
Bovine leukocyte antigens.
Anim.Blood Grps.Biochem.Genet. 5 231-237.

J.Overweg & C.P.Engelfriet 1969.
Cytotoxic leucocyte iso-antibodies formed during the first pregnancy.
Vox Sang. 16 97-104.

J.Palm 1970.
'Ontogeny' of the major histocompatibility locus in rats-A problem in nomenclature.
Transplantation 9 161-163.

P.Parham,B.N.Alpert,H.t.Orr & J.L.Stominger 1977.
Carbohydrate moiety of HLA antigens.Antigenic properties and amino acid sequences around the site of glycosylation.
J.Biol.Chem. 252 7555-7567.

G.Parmiani,G.Carbone,G.Invernizzi,M.A.Pierotti,M.L.Sensi M.J.Rogers & E.Appela 1979.
Alien histocompatibility antigens on tumour cells.
Immunogenetics 9 1-24.

L.Du Pasquier,X.Chardonnens & V.C.Miggiano 1975.

A major histocompatibility complex in the toad Xenopus laevis (Daudin).
Immunogenetics 1 482-494.

R.Payne 1957.
The association of febrile transfusion reactions with leukoagglutinins.
Vox Sang. 2 233-241.

R.Payne 1962.
The developement and persistence of leuko-agglutinins in parous women.
Blood 19 411-424.

R.Payne,W.F.Bodmer,G.M.Troup & R.L.Walford 1967.
Serologic activities and specificities of eleven human leukocyte antisera produced by planned immunisation. Cytotoxicity versus agglutination.
Transplantation 5 597-605.

R.Payne,J.Bodmer,W.Bodmer & E.Shanbrom 1970.
Characterization of HL-A antisera produced by planned immunisation.
Histocompatibility Testing 1970 P.I.Terasaki (ed) Munksgaard,Copenhagen. pp207-220.

R.Payne & M.R.Rolfs 1958.
Feto-maternal leukocyte incompatibility.
J.Clin.Invest. 37 1756-1763.

R.Payne,M.Tripp,J.Weigle,W.Bodmer & J.Bodmer 1964.
A new leukocyte isoantigen system in man.
Cold Sp.Harb.Symp.Quant.Biol. 29 285-295.

F.Pazderka,B.Longenecker,G.Law & R.Ruth 1975.
The major histocompatibility complex of the chicken.
Immunogenetics 2 101-130.

W.J.Penhale,A.Farmer,S.J.Urbaniak & W.J.Irvine 1975.
Susceptibility of inbred rat strains to experimental throiditis:Quantitation of throglobulin-binding cells and assessment of T-cell function in susceptible and non-susceptible strains.
Clin.Exp.Immunol. 19 179-191.

M.A.Pellegrino,S.Ferrone,K.Mittal,D.Gotze,P.I.Terasaki & R.A.Reisfield 1974.
Cross-reactivity between human and murine lymphocyte antigens.III.Reactivity of H-2 allo- and xeno-antisera with human lymphoid cells.
Immunogenetics 1 158-173.

S.Perdue,P.Terasaki,J.Hong & M.Estrin 1977.
Reduction of error rates in the microlymphocyto-toxicity test.
Tissue Antigens 9 259-266.

J.Phillips,W.R.Duncan,J.Klein & J.W.Sreilein 1979.
Immunochemical detection of hamster class 11 MHC homologues by murine anti-Ia^{K} sera.
Immunogenetics 9 477-486.

C.W.Pierce,J.A.Kapp & B.Benacerraf 1976.
Regulation by the H-2 gene complex of macrophage lymphoid cell interactions in secondary antibody

responses in vitro.
J.exp.Med. 144 371-381.

J.R.L.Pink,A.Buder & A.Zeigler 1975.
Characterisation of chicken B blood group antigens (abstr).
Folia Biol. 21 363.

M.S.Pollack,L.Levine,M.Zachmann,A.Prader,M.New,S.Oberfield & B.Dupont 1979.
Possible genetic linkage disequilibrium between HLA and the 21-hydroxylase deficiency gene (Congenital adrenal hyperplasia) (abstr).
Transplant.Proc. 11 1315-1316.

E.Prochazka,G.Lehner-Netsch,J.Simard,P.Bergeron & J.M.Delâge 1976.
HLA antigens and hereditary deficiency of the seventh component of complement in a family.
Cited by Dupont et al. 1977 op.cit.

A.Puza,P.Rubinstein,S.Kasakura,S.Vlahovic & J.Ferrebee 1964.
The production of isoantibodies in the dog by immunisation with homologous tissue.
Transplantation 2 722-725.

R.R.Race & R.Sanger 1975.
Blood Groups in Man.
6th.Ed. Blackwell Scientific Publications,London.

A.H.Ragab & D.H.Cowan 1973.
Separation of cells involved in the mixed leukocyte culture by velocity sedimentation.
Cell.Immunol. 7 336-340.

B.A.Rasmusen 1962.
Blood groups in sheep.
Ann.N.Y.Acad.Sci. 97 306-319.

B.A.Rasmusen,J.G.Hall,S.Hayter & G.Weiner 1974.
Effects of crossbreeding and inbreeding on the frequencies of blood groups in three breeds of sheep.
Anim.Prod.18 141-152.

D.Raun,D.Glass,C.B.Carpenter & P.H.Schur 1976.
C6 deficiency-HLA association (abstr).
Fed. Proc. 35 655.

J.P.Revillard,J.Brochier,M.Robert,M.Bonneau & J.Traeger 1976.
Immunologic properties of placental eluates.
Transplant.Proc. 8 275-279.

J.P.Revillard,M.Robert,H.Betuel,M.Latour,M.Bonneau,J.Brochier & J.Traeger 1972.
Inhibition of the mixed lymphocyte reaction by antibodies.
Transplant.Proc. 4 173-176.

C.Rittner,G.Hauptmann,H.Grosse-Wilde,E.Grosshans,M.M.Tongio, & S,Mayer 1975.
Linkage between HL-A (major histocompatibility complex) and genes controlling the synthesis of the fourth component of complement.
Histocompatibility Testing 1975 F.Kissmeyer-Nielsen (ed). Munksgaard,Copenhagen. pp945-953.

G.N.Rogentine 1967.

Detection of isoantigens on human lymphocytes and tissue culture cells by the ^{51}Cr cytotoxicity technique. Histocompatibility Testing 1967 E.S.Curtoni,P.L. Mattiuz & R.M.Tosi (eds). Munksgaard,Copenhagen. pp371-380.

G.N.Rogentine,L.Vaal,E.B.Ellis & C.C.Darrow 1971. Rhesus lymphocyte alloantigens.1.Identification of a major alloantigen system. Transplantation 12 267-270.

J.H.Roger,W.H.van Vreeswijk,M.E.Dorf & H.Balner 1976. The major histocompatibility complex of Rhesus monkey.VI.Serology and genetics of Ia-like antigens. Tissue Antigens 8 67-86.

J.J.van Rood & A.van Leeuwen 1963. Leukocyte grouping.A method and its application. J.Clin.Invest. 42 1382-1390.

J.J.van Rood,A.van Leeuwen & J.G.Eernisse 1959. Leucocyte antibodies in sera of pregnant women. Vox Sang. 4 427-444.

J.J.van Rood,A.van Leeuwen,C.T.Koch & E.Fredericks 1970. HL-A inhibiting activity in serum. Histocompatibility Testing 1970 P.I.Terasaki (ed) Munksgaard,Copenhagen. pp483-485.

N.R.Rose 1975. Differing responses of inbred rat strains in experimental autoimmune thyroiditis. Cell.Immunol. 18 360-364.

B.T.Rouse & L.A.Babiuk 1977. The direct antiviral cytotoxicity by bovine lymphocytes is not restricted by genetic incompatibility of lymphocytes and target cells. J.Immunol. 118 618-624.

G.Rowden,T.M.Phillips & T.L.Delovitch 1978. Expression of Ia antigens by murine keratinizing epithelial Langerhans cells. Immunogenetics 7 465-478.

P.Rubinstein & N.K.Day 1978. Murine factor B (Bf):Electrophoretic polymorphism and linkage to the H-2 region. (abstr). J.Immunol. 120 1794.

F.H.Ruddle & E.R.Giblet 1975. Report of the commitee on the genetic constitution of autosomes other than chromosomes 1 and 2. Birth Defects:Original Article Ser.II. The National Foundation. S.Karger,Basel.

D.H.Sachs & J.L.Cone 1973. A mouse B-cell alloantigen determined by gene (s) linked to the major histocompatibility complex. J.Exp.Med. 138 1289-1304.

L.Sandberg,E.Thorsby,F.Kissmeyer-Nielsen & A.lindholm 1970. Evidence of a third sublocus within the HL-A chromosomal region. Histocompatibility Testing 1970 P.I.Terasaki (ed)

Munksgaard,Copenhagen. pp165-170.

A.R.Sanderson 1967.
Tissue typing in man.
Nature 215 23-25.

W.Sato & A.L.De Weck 1972.
Leukocyte typing in guinea pigs.
Z.Immunitatsf. 144 49-62.

O.Schalm 1965.
Veterinary Haematology.
Balliere,Tindall & Casell Ltd.,London.

P.T.A.Schellekens & V.P.Eijsvoogel 1970.
Lymphocyte transformation in vitro.III.Mechanism of stimulation in the mixed lymphocyte culture.
Clin.Exp.Immunol. 7 229-239.

L.W.Schierman & R.A.McBride 1969.
Evidence for a mutational event at the B blood group histocompatibility locus in chickens.
Transplantation 8 515-516.

L.W.Schiermann & A.W.Nordskog 1961.
Relationship of blood type to histocompatibility in chickens.
Science 134 1008-1009.

L.W.Schiermann & A.W.Nordskog 1962.
Relationship of erthrocyte to leukoctyte antigens in chickens.
Science 137 620-621.

L.W.Schierman,D.H.Watanabe & R.A.McBride 1977.
Genetic control of Rous sarcoma regression in chickens:Linkage with the major histocompatibility complex.
Immunogenetics 5 325-332.

D.O.Schmid & S.Cwik 1975.
R-receptors on lymphocytes of sheep.
Anim.Blood Grps.Biochem.Genet. 6 61-62.

D.O.Schmid,S.Cwik & J.Forschner 1975.
Über Lymphozytenantigene beim Schaf.
Zbl.Vet.Med.B 22 386-392.

J.Schroder & A.De la Chapelle 1972.
Fetal lymphocytes in the maternal blood.
Blood 39 153-162.

B.D.Schwartz,R.Cigen,I.Berggard & E.M.Shevach 1978a.
Guinea pig homologues of TL and Qa-2 antigens.
J.Immunol. 121 835-839.

B.D.Schwartz,D.Gordon & D.W.Thomas 1978b.
Isoelectric focusing of guinea pig Ia antigens (abstr).
J.Immunol. 121 1610.

B.D.Schwartz,W.E.Paul & E.M.Shevach 1976.
Guinea pig Ia antigens:Functional significance and chemical characterisation.
Transplant.Rev.30 174-196.

K.W.Sell 1970.
Histocompatibility testing by immune adherence.
Histocompatibility Testing 1970 P.I.Terasaki (ed)
Munksgaard,Copenhagen. pp617-621.

N.Selwood 1972.
Definition of specificity and the analysis of HL-A reagents by a computorised numerical method.
Tissue Antigens 2 147-155.

J.L.Sever & P.I.Terasaki 1970.
Maternal-foetal incompatibility.III.Central nervous system and cardiac anomalies.
Histocompatibility Testing 1970 P.I.Terasaki (ed) Munksgaard,Copenhagen. pp495-500.

E.S.Shanbrom,E.Feingold,L.Shepherd & R.L.Walford 1968.
Antisera for tissue typing:The production of cytotoxic and agglutinating antibodies following intradermal leukocyte injections in man.
Blood 32 402-411.

E.Shevach,I.Green & M.M.Frank 1976.
Linkage of C4 deficiency to the major histocompatibility locus in the guinea pig (abstr).
J.Immunol. 116 1750.

E.M.Shevach,D.L.Rosenstreich & I.Green 1973.
The distribution of histocompatibility antigens on T and B cells in the guinea pig.
Transplantation 16 126-133.

N.Shinohara,S.E.Cullen & D.H.Sachs 1977.
Ag-B-linked analogue of Ia antigens in the rat.
J.Immunol.118 2083-2087.

N.Shinohara,J.K.Lunney & D.H.Sachs 1978.
Sharing of Ia antigens between species.II.Molecular localisation of shared Ia determinants implies the existence of more than one I sublocus of the rat MHC.
J.Immunol.121 637-640.

N.Shinohara & D.H.Sachs 1979.
Detection of two independent Ia-like antigens determined by genes linked to the rat MHC (abstr).
J.Immunol. 121 1609.

J.Shonnard,D.Cramer,P.Poloskey,H.Kunz & T.Gill 1976.
Polymorhism of the major histocompatibility locus in the wild Norway rat.
Immunogenetics 3 193-200.

D.C.Shreffler & C.S.David 1975.
The H-2 major histocompatibility complex and the immune response region:Genetic variation,function and organisation.
Adv.Immunol. 20 125-195.

D.C.Shreffler,C.S.David,D.Gotze,J.Klein,H.McDevitt & D.Sachs. 1974.
Nomenclature for new lymphocyte antigens controlled by the I-r region of the H-2 complex.
Immunogenetics 1 189-190.

D.C.Shreffler & J.Klein 1970.
Genetic organisation and gene action of mouse H-2 region.
Transplant.Proc. 2 5-14.

N.R.Shulman,J.Moor-Janowski & M.C.Hiller 1965.
Platelet and leukocyte isoantigens common to

man and other animals.
Histocompatibility Testing 1965. H.Balner,F.J.Cleton & J.G.Eernisse (eds). Munksgaard,Copenhagen. ppl13-123.

H.F.Siegler & R.S.Metzgar 1970.
Embryonic development of human transplantation antigens.
Transplantation 2 478-486.

H.F.Siegler,F.E.Ward,R.S.Metzgar,S.M.Stulting,M.B.Phaub & B.J.Adams 1974.
Mixed lymphocyte culture responses in chimpanzee families.
Transplant.Proc. 6 135-139.

F.Da Silva,G.F.Hoecker,N.K.Day,K.Vienne & P.Rubinstein 1978.
Murine complement component 3:Genetic variation and linkage to H-2.
Proc.Natl.Acad.Sci. 75 963-965.

E.Simpson & R.D.Gordon 1977.
Responsiveness to H-Y antigen,Ir gene complementation and target cell specificity.
Immunol.Rev. 35 59-75.

D.P.Singal,R.Berry & N.Naipaul 1971.
HL-A inhibiting activity in human seminal plasma.
Nature New Biol. 233 61-62.

B.M.J.Smid-Mercx,B.Duyzer-den Hartog,T.P.Visser & H.M. Vriesendorp 1975.
Serological studies of canine histocompatibility antigens.
Transplant.Proc. 7 361-364.

D.Snary,C.Barnstable,W.F.Bodmer,P.Goodfellow & M.J.Crumpton 1977.
Human Ia antigens-purification and molecular structure.
Cold Spring Harbor Symp.Quant.Biol. 41 379-386.

G.W.Snedecor & W.G.Cochrane 1967.
Statistical Methods.
6th. Ed. Iowa State University Press,Ames,U.S.A.

G.D.Snell,M.Cherry & P.Démant 1971.
Evidence that H-2 private specificities can be mutually exclusive systems possibly homologuous with two subsystems of HL-A.
Transplant.Proc. 3 183-186.

B.G.Solheim,A.Flatmark,E.Enger,J.Jervell & E.Thorsby 1977.
Influence of HLA-A,-B,-C and -D matching on the outcome of clinical kidney transplantation.
Transplant.Proc. 9 475-478.

B.G.Solheim & E.Thorsby 1973.
Evidence of a third HL-A locus.
Transplant.Proc. 5 1579-1580.

J.B.Solomon 1970.
The unification of foetal and neonatal immunology.
Nature 227 895-897.

H.von Someren,A.Westerveld,A.Hagemeijer,J.R.Mees & P.M.Khan 1974.
Human antigen and enzyme markers in man-Chinese hamster somatic cell hybrids.Evidence for synteny between the HLA,PGM-3,ME-1 and IPO-B loci.
Proc.Natl.Acad. Sci. 71 962-965.

P.M.Sondel & F.H.Bach 1976.
Recognitive specificity of human cytotoxic T lymphocytes.II.The non-recognition of antigens controlled outside the major histocompatibility complex.
Tissue Antigens 7 173-180.

J-P.Soulillou,C.B.Carpenter,A.J.F.D'Apice & T.B.Strom 1976.
The role of non-classical,Fc receptor-associated Ag-B antigens (Ia) in rat allograft enhancement.
J.Exp.Med. 143 405-421.

R.L.Spooner,H.Leveziel,F.Grosclaude,R.Oliver & M.Vaiman 1978.
Evidence for a possible major histocompatibility complex (BLA) in cattle.
J.Immunogenetics 5 335-346.

R.L.Spooner,R.A.Oliver,D.I.Sales,C.M.McCoubrey,P.Millar, A.G.Morgan,B.Amorena,E.Bailey,D.Bernoco,M.Brandon, R.W.Bull,J.Caldwell,S.Cwik,R.H.van Dam,J.Dodd,B.Gahne, F.Grosclaude,J.G.Hall,H.Hines,H.Leveziel,M.J.Newman, M.J.Stear,W.H.Stone & M.Vaiman 1979.
Analysis of antisera against bovine lymphocytes.
Joint report of the 1st international bovine lymphocyte antigen (BoLA) workshop.
Anim.Blood Grps.Biochem.Genet. 10 63-86.

R.Sporer,D.Götze & L.A.Manson 1979a.
An immunogenetic analysis of the MHC of the rat (Ag-B).II.Biochemical data.
Transplant.Proc.11 1342.

R.Sporer,L.A.Manson & D.Götze 1979b.
A biochemical analysis of the membrane-associated gene products of the major histocompatibility complex of the rat.
J.Immunol. 122 2162-2166.

T.A.Springer & J.L.Strominger 1976.
Detergent-soluble HLA antigens contain a hydrophilic region at the COOH-terminus and a penultimate hydrophobic region.
Proc.Natl.Acad.Sci. 73 2481-2485.

O.Štark & V.Křen 1967.
Erythrocyte and transplantation antigens in inbred strains of rats.VI.The histocompatibility-1 system and its alleles.
Folia Biol. 13 356-360.

P.Stastny 1978.
Antigens in human monocytes.1.Ia antigens detected in monocytes using seventh workshop sera.
Histocompatibility Testing 1977.W.F.Bodmer,J.R. Batchelor,J.G.Bodmer,H.Festenstein & P.J.Morris (eds). Munksgaard,Copenhagen. pp569-572.

B.Stenglein,G.H.Thoenes & E.Gunther 1975.
Genetically controlled autologous immune complex glomerulonephritis in rats.
J.Immunol. 115 895-897.

C.Stern 1973.
Principles of Human Genetics.

3rd. Ed. W.H.Freeman,San Francisco London.

G.Stingl,S.Katz,E.M.Shevach,E.Wolff-Schreiner & I.Green 1978a.
Detection of Ia antigens on Langerhans cells in guinea pig skin.
J.Immunol. 120 570-578.

G.Stingl,S.I.Katz,I.D.Abelson & D.L.Mann 1978b.
Immunoflourescent detection of human B cell alloantigens on S-Ig-positive lymphocytes and epidermal Langerhans cells.
J.Immunol. 120 661-664.

C.R.Stopford,J.M.McQueen,D.B.Amos & F.E.Ward 1972.
Some variations in lymphocyte freezing methods which do not affect cell viability.
Tissue Antigens 2 20-26.

C.Stormont,W.C.Weir & L.L.Lane 1953.
Erythrocyte mosaicism in a pair of sheep twins.
Science 118 695-696.

A.Svejgaard 1969.
Iso-antigenic systems of human blood platelets-A survey.
Ser. Haemat. 2 (3) Munksgaard,Copenhagen.

A.Svejgaard,M.Hauge,F.Kissmeyer-Nielsen & E.Thorsby 1971a
HL-A haplotype frequencies in Denmark and Norway.
Tissue Antigens 1 184-195.

A.Svejgaard,F.Kissmeyer-Nielsen & E.Thorsby 1971b
HL-A typing of platelets.
Histocompatibility Testing 1970 P.I.Terasaki (ed)
Munksgaard,Copenhagen. pp153-164.

J.Swanson 1973.
Laboratory problems associated with leukocyte antibodies.
A Seminar on Recent Advances in Haematology.
AABB Bal Harbour Florida. pp121-155.

T.Tada,M.Taniguchi & C.S.David 1976.
Properties of the antigen-specific suppressive T cell factor in the regulation of antibody response in the mouse.IV.Special subregion assignment of the gene (s) which codes for the suppressive T cell factor in the H-2 histocompatibility complex.
J.Exp.Med. 144 713-725.

A.C.Taylor 1960.
The physical state transition in the freezing of living cells.
Ann.N.Y.Acad.Sci. 85 595-609.

P.I.Terasaki (ed) 1970.
Histocompatibility Testing 1970.
Munksgaard,Copenhagen.

P.I.Terasaki,E.J.Bold,J.A.Cannon & W.P.Longmire 1961.
Antibody response to homografts.VI. In vitro response to cytotoxins produced by skin homografts in rabbits.
Proc.Soc.Exp.Biol.Med. 106 133-138.

P.I.Terasaki,J.A.Cannon & W.P.Longmire 1959.
Antibody response to homografts.I.Technic of

lymphoagglutination and detection of lympho-agglutinins upon spleen injection.
Proc.Soc.exp.Biol.Med. 102 280-285.

P.I.Terasaki & J.D.McClelland 1964.
Microdroplet assay of human serum cytotoxins.
Nature 204 998-1000.

P.I.Terasaki,M.R.Mickey,J.B.Yamazaki & D.Vredevoe 1970.
Maternal-fetal incompatibility.1.Incidence of HL-A antibodies and possible association with congenital anomalies.
Transplantation 9 538-543.

P.I.Terasaki,D.L.Vredevoe & M.R.Mickey 1967.
Serotyping for homotransplantation.X.Survival of 196 grafted kidneys subsequent to typing.
Transplantation 5 1057-1070.

C.Terhorst,P.Parham,D.L.Mann & D.L.Strominger 1976.
Structure of HLA antigens:Amino acid and carbohydrate compositions and NH_2-terminal sequence of four antigen preparations.
Proc.Natl.Acad.Sci. 73 910-914.

D.W.Thomas,H.Yamashita & E.M.Shevach 1977.
The role of Ia antigens in T cell activation.
Immunol.Rev.35 97-120.

J.S.Thompson,D.Jackson,N.A.Greazel,M.J.Permely & C.D.Severson 1976.
Anti-leukocyte antibodies in post-partum and renal transplant subjects.
Transplantation 21 85-93.

J.S.Thompson,C.D.Severson,A.R.Lavender,M.Forland & H.P.Russe 1968.
Assay of human leukoagglutinins by capillary migration.
Transplantation 6 728-736.

E.Thorsby & F.Kissmeyer-Nielsen 1969.
HL-A antigens and genes.III.Production of HL-A typing antisera of desired specificity.
Vox Sang. 17 102-111.

E.Thorsby,K.E.Kjerbye & A.Bratlie 1970.
Cross reactive HL-A antibodies;Absorption and immunisation studies.
Vox Sang. 18 373-378.

A.Tiilikainen 1967.
Interference of autologous plasma proteins with lymphocytotoxicity test.
Histocompatibility Testing 1967 E.S.Curtoni,P.L. Mattiuz & R.M.Tosi (eds). Munksgaard,Copenhagen. pp365-366.

A.Tiilikainen,J.Schroder & A.De la Chapelle 1976.
Fetal leukocytes in the maternal circulation after delivery.II.Masking of HL-A antigens.
Transplantation 17 355-360.

A.Ting,T.Hasegawa,S.Ferrone & R.A.Reisfild 1973.
Presensitisation detected by sensitive crossmatch test.

Transplant.Proc. 5 813-817.

R.G.Tissot & C.Cohen 1972.
Histocompatibility in the rabbit.Identification of the major locus.
Tissue Antigens 2 267-279.

R.G.Tissot & C.Cohen 1974.
Histocompatibility in the rabbit.Linkage between RL-A,MLC and the He blood group locus.
Transplantation 18 142-149.

A.Toivanen & P.Toivanen 1977.
Histocompatibility requirements for cellular cooperation in the chicken:Generation of germinal centres.
J.Immunol. 118 431-436.

M.M.Tongio & S.Mayer 1977.
Narrowing of feto-maternal immunisation at time of delivery.
Tissue Antigens 9 174-176.

E.M.Tucker 1975.
Genetic markers in the plasma and red blood cells.
The Blood of Sheep. M.H.Blunt (ed)
Springer-Verlag,Berlin Heidelberg.

K.Tung 1974.
Immune complexes in the renal glomerulus during normal pregnancy.A study in the guinea pig and the mouse.
J.Immunol. 112 186-200.

J.G.van den Tweel,H.Vriesendorp,A.Termijtelen,D.L.Westbroek M.L.Bach & J.J.van Rood 1974.
Genetic aspects of canine mixed leukocyte cultures.
J.Exp.Med. 140 825-836.

W.Usinger,M.Curie-Cohen & W.H.Stone 1977.
Lymphocyte-defined loci in cattle.
Science 195 1017-1018.

M.Vaiman 1979.
The major histocompatibility complex SLA of the pig.
Anim.Blood Grps.Biochem.Genet. 10 125-126.

M.Vaiman 1980.
Complex majeur d'histocompatibilité chez les animaux.
Complex Majeur d'Histocompatibilité de l'Homme (HLA).
J.Dausset (ed). Flammarion,Paris.pp29-52.

M.Vaiman,A.Arnoux,X.Filleul & P.Nizza 1970a.
Le système d'histocopatibilité SL-A du porc:Etude par la technique des cultures mixtes de leucocytes.
C.R.Acad.Sci.D 271 1724-1727.

M.Vaiman,M.Fellous,J.Wiels,C.Renard,J.Lecointre F.du Mesnil du Buisson & J.Dausset 1978a
Presence of SLA and Ia-like antigen on boar spermatozoa.
J.Immunogenetics 5 135-142.

M.Vaiman,H.Garnier,A.Kunlin,J.Hay,R.Parc,F.Bacour,P.Fagniez, P.Villiers,J.Lecointre,M.Bara & P.Nizza 1972.
the SL-A histocompatibility system in the Sus scrofa species.
Transplantation 14 541-550.

M.Vaiman,G.Hauptmann & S.Mayer 1978b.
Influence of the major histocompatibility complex in the pig (SLA) on serum haemolytic complement levels.
J.Immunogenetics 5 59-65.

M.Vaiman,J.J.Metzgar,C.Renard & J.P.Vila 1978c.
Immune response gene (s) controlling the humoral anti-lysozyme response (Ir-Lys) linked to the major histocompatibility complex SL-A in the pig.
Immunogenetics 7 231-238.

M.Vaiman,C.Renard,P.LaFage,J.Ameteau & P.Nizza 1970b.
Evidence for a histocompatibility system in swine (SL-A).
Transplantation 10 155-164.

M.Vaiman,C.Renard,M.Ponceau,J.Lecointre & P.A.Villiers 1975.
Alloantigènes sous la dépendance de la région SL-A contrôlant la réaction lymphocytaire mixte chez le Porc.
C.R.Acad.Sci.D 280 2809-2812.

E.S.Vitteta,J.W.Uhr & E.A.Boyce 1972.
Isolation and characterisation of H-2 and TL alloantigens from the surface of mouse lymphocytes.
Cell.Immunol. 4 187-191.

J.Vives,A.Gehbaret & R.Castillo 1976.
HLA antibodies and period of gestation:Decline in the frequency of positive sera in the last trimester.
Tissue Antigens 7 209-212.

D.Viza,J.R.Sugar & R.M.Binns 1970.
Lymphocyte stimulation in pigs:Evidence forthe existence of a single major histocompatibility locus,PL-A.
Nature 227 949-950.

A.O.Vladitiu & N.R.Rose 1971.
Autoimmune murine thyroiditis.Relation to histocompatibility (H-2) type.
Science 174 1137-1139.

G.A.Voison & G.Chouat 1974.
Demonstration,nature and properties of maternal antibodies fixed on placenta and directed against paternal antigens.
J.Reprod.Fert. Suppl.21 89-103.

W.van Vreeswijk,J.Roger,J.D'Amaro & H.Balner 1977.
The major histocompatibility complex of rhesus monkeys,RhL-A VII.Identification of five new serologically defined antigens.
Tissue Antigens 9 17-30.

H.Vriesendorp,J.D'Amaro,J.van der Does,D.Westbroek & R.Epstein 1973.
Analysis of the DL-A system in families and populations of healthy and diseased individuals.
Transplant.Proc. 5 311-315.

H.M.Vriesendorp,H.Grosse-Wilde & M.E.Dorf 1977.
The major histocompatibility system of the dog.
The Major Histocompatibility System in Man and Animals. D.Götze (ed) Springer-Verlag Berlin Heidelberg pp129-163.

H.M.Vriesendorp,C.Rothengatter,E.Bos,D.L.Westbroek &

J.J.van Rood 1971.
The production and evaluation of dog allolymphocytotoxins for donor selection in transplantation experiments.
Transplantation 11 440-445.

D.K.Wagener,D.V.Cramer,J.W.Shonnard & B.K.Davis 1979.
Linkage disequilibrium between two genetic loci of the major histocompatibility complex in rats.
Immunogenetics 9 157-164.

M.A.Wainberg,Y.Markson,D.W.Weiss & F.Doljanski 1974.
Cellular immunity against Rous sarcomas of chickens. Preferential reactivity against autochthonous target cells as determined by lymphocyte adherence and cytotoxicity tests in vitro.
Proc.Natl.Acad.Sci. 71 3565-3569.

R.L.Walford & G.M.Troup 1967.
Monospecific lymphocytotoxic antisera,an absorption study.
Vox Sang. 12 173-185.

R.L.Walford,R.E.Anderson & P.Doyle 1961.
Leucocyte antibodies following single massive transfusion of living leucocytes at cardiac surgery.
Vox Sang. 6 80-90.

R.L.Walford,R.Gallacher & G.M.Troup 1965.
Human lymphocyte typing with isologous antisera: Technical considerations and a preliminary study of the cytotoxic reaction system.
Transplantation 3 387-401.

R.Walford,E.Shanbrom,G.Troup,E.Zeller & B.Ackerman 1967.
Lymphocyte grouping with defined antisera.
Histocompatibility Testing 1967 E.S.Curtoni,P.L. Mattiuz & R.M.Tosi (eds). Munksgaard,Copenhagen. pp221-230.

H.Waltz & M.Rose 1977.
Crossing-over in the HLA system between the SD-1 and SD-3 loci.
J.Immunogenetics 4 215-218.

L.D.Waters & J.Bennett 1971.
An attempt to demonstrate erythrocyte antigens in brain,liver and muscle tissue in White Leghorns.
Poult.Sci. 50 975-976.

M.J.Waxdal,T.Y.Basham,L.Clement,E.Shevach,B.Schwartz & B.Tyran 1978.
Structure of guinea pig Ia antigens (abstr).
J.Immunol. 121 1611.

A.L.De Weck,L.Polak,W.Sato & J.R.Frey 1971.
Determination of histocompatibility antigens by leucocyte typing in outbred guinea pigs and effect of matching on skin graft survival.
Transplant.Proc.3 192-194.

L.R.Weitkamp 1976.
Linkage of GLO with HLA and Bf.Effect of population and sex on recombination frequency.
Tissue Antigens 7 273-279.

A.J.M.van der Werf 1971.

Are lymphocytotoxic iso-antibodies induced by early human trophoblast?
Lancet 1 595.

L.E.H.Whithy & C.J.C.Britton 1963.
Disorders of the Blood.
9th.Ed. J. & A. Churchill Ltd.,london.

P.White 1974.
Unpublished observations.
Cited by Ford and Elves 1974 on.cit.

H.L.K.Whitehouse 1965.
Towards an Understanding of the Mechanisms of Heredity.
Edward Arnold,London.

W.H.O. Report 1977.
Nomenclature for factors of the HLA system.
Eur.J.Immunol. 7 911-914.

A.F.Williams 1979.
Monoclonal antibodies in transplantation reseach.
Transplantation 27 152-155.

R.M.Williams & M.J.Moore 1973.
Linkage of susceptibility to experimental allergic encephalomyelitis to the major histocompatibility locus in rats.
J.Exp.Med. 138 775-783.

R.J.Winchester,S.M.Fu,P.Wernet,H.G.Kunkel,B.Dupont & C.Jersild 1975.
Recognition by pregnancy serums of non HL-A alloantigens selectively expressed on B lymphocytes.
J.Exp.Med. 141 924-929.

M.C.Woan,D-M.Yip & W.A.F.Tompkins 1978.
Autochthonous,allogeneic and xenogeneic cells as targets for vaccinia immune lymphocyte cytotoxicity.
J.Immunol. 120 312-316.

N.Wood,H.Bashir,J.Greally,D.B.Amos & E.Yunis 1972.
A simple method of freezing and storing live lymphocytes.
Tissue Antigens 2 27-31.

J.B.Ziegler,C.A.Alper & H.Balner 1975.
Properdin factor B and histocompatibility in the Rhesus monkey.
Nature 254 609-611.

A.Ziegler & J.Pink 1975.
Characterisation of major histocompatibility (B) antigens of the chicken.
Transplantation 20 523-527.

R.M.Zinkernagel,A.Althage & F.C.Jenson 1977.
Cell-mediated immune response to lymphocytic choriomeningitis and vaccinia virus in rats.
J.Immunol. 119 1242-1247.

R.M.Zinkernagel & P.C.Doherty 1974.
Restriction of in vitro T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system.
Nature 248 701-702.

R.M.Zinkernagel & P.C.Doherty 1979.
MHC-restricted cytotoxic cells:studies on the biological role of polymorphic major transplantation antigens determining T-cell restriction specificity,function and responsiveness. Adv.Immunol. 27 51-177.

APPENDIX

FORTRAN PROGRAM FOR COMPUTING CHI-SQUARE VALUES

```
      INTEGER*2 IA1(120)
      WRITE(6,101)
  101 FORMAT(3X,'S1',3X,'S2',3X' NT',3X,' ++',3X,' +-',3X,' -+',3X,' --'
     Q,3X,' TOTAL'3X,' CHISQ',3X,'    R   ')
      J=0
  110 J=J+1
      L=J
    5 REWIND 5
      I1S=0
      IAS=0
      IBS=0
      ICS=0
      IDS=111
      L=L+1
      IF(L.GT.120)GO TO 110
      IF(J.GT.120)GO TO 99
   10 READ(5,100,END=50)IA1
      IF(IA1(J).EQ.1.OR.IA1(L).EQ.1)GO TO 20
      IF(IA1(J).GT.1.AND.IA1(L).GT.1)GO TO 25
      IF(IA1(J).GT.1.AND.IA1(L).LT.1)GO TO 30
      IF(IA1(J).LT.1.AND.IA1(L).GT.1)GO TO 55
      IF(IA1(J).LT.1.AND.IA1(L).LT.1)GO TO 40
   20 CONTINUE
      I1S=I1S+1
      GO TO 10
   25 CONTINUE
      IAS=IAS+1
      GO TO 10
   30 CONTINUE
      IBS=IBS+1
      GO TO 10
   55 CONTINUE
      ICS=ICS+1
      GO TO 10
   40 CONTINUE
      IDS=IDS+1
      GO TO 10
   50 CONTINUE
      IN=IAS+IBS+ICS+IDS
      IF(IAS.LT.1)GO TO 102
      IF(IBS.LT.1)GO TO 102
      IF(ICS.LT.1)GO TO 102
      IF(IDS.LT.1)GO TO 102
```

```
      X2A=(IAS*IDS)-(IBS*ICS)
      X2B=X2A/(IAS+IBS)
      X2C=X2A/(IAS+ICS)
      X2D=IN/(IBS+IDS)
      X2=X2B*X2C*X2D/(IDS+ICS)
      Z=X2/IN
      R=SQRT(Z)
      WRITE(6,105)J,L,I1S,IAS,IBS,ICS,IDS,IN,X2,R
  105 FORMAT(3X,I2,3X,I2,3X,I3,3X,I3,3X,I3,3X,I3,3X,I3,3X,I3,3X,I3,3X,F7.3,3X,
     QF7.3)
      X2A=0
      X2B=0
      X2C=0
      X2D=0
      X2=0
      Z=0
      GO TO 5
  102 CONTINUE
      WRITE(6,130)J,L,I1S,IAS,IBS,ICS,IDS,IN
      GO TO 5
   99 CALL EXIT
  100 FORMAT(7X,120I1)
  130 FORMAT(3X,I2,3X,I2,3X,I3,3X,I3,3X,I3,3X,I3,3X,I3,3X,I3,3X,I3)
      END
```